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- (71) Applicant (for all designated States except US): UNIVERSITY OF SOUTH FLORIDA [US/US]; 4202 East Fowler Avenue, FAO 126, Tampa, FL 33620-7900 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): PHILLIPS, Ian, M. [US/US]; 5150 Royal Cypress Circle, Tampa, FL 33647 (US). TANG, Yao, Liang [CN/US]; 4892 Beach Dr. SE, Apt.C, St. Petersburg, FL 33705 (US).
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(54) Title: VIGILANT CELLS

(57) Abstract: Bone marrow-derived mesenchymal stem cells were transduced with a stimulus-responsive rAAV vector system that detects and responds to hypoxia in cardiac tissue. A first rAAV vector in the system contains a cardiac-specific promoter linked to a sequence encoding an oxygen-sensitive chimeric transactivator containing a GAL4 DNA binding domain, an oxygen-dependent degradation domain, and a p65 activation domain. A second rAAV vector contains a cardioprotective gene linked to a GAL4 UAS. The first rAAV vector expresses the chimeric transactivator specifically in the heart, and in response to hypoxia, the transactivator binds to the GAL4 upstream activating sequence (UAS) in the second rAAV vector. Binding of the transactivator to the UAS results in the expression of the cardioprotective gene. The rAAV vectors can be used to treat cells in a number of other disease states, including diabetes, cancer, stroke, and atherosclerosis. These approaches to stem cell-based gene therapy provide a novel strategy not only for treatment but for prevention of cell destruction.

DESCRIPTION

VIGILANT CELLS

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Cross-Reference to Related Applications

The present application claims benefit of U.S. Provisional Application Serial No. 60/513,657, filed October 23, 2003, U.S. Provisional Application Serial No. 60/494,185, filed August 11, 2003, U.S. Provisional Application Serial No. 60/513,067, filed October 21, 2003, and U.S. Provisional Application Serial No. 60/494,184, filed August 11, 2003, each of which are hereby incorporated by reference herein in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, and drawings.

Government Support

15 The subject matter of this application has been supported by a research grant from the National Institutes of Health under grant number 5R37HL2733423. Accordingly, the government may have certain rights in this invention.

Background of the Invention

20 Stem cell-based therapies are being explored for the treatment of several disease states characterized by damaged tissue. Although stem cells injected into damaged tissue have been shown to at least partially regenerate the tissue, the delivered cells and/or regenerated tissue are subsequently destroyed by the underlying cause of the disease. The development of methods for enhancing the survival of transplanted stem cells/regenerated tissue would therefore be useful for the successful implementation of stem cell regenerative therapy.

Life-threatening and chronic diseases are often characterized by the presence of particular physiological signals. For example, hypoxia is a signal associated with ischemic heart disease, while high blood glucose is a signal for diabetes. Such disease-associated signals directly or indirectly modulate gene expression in cells exposed to the signals. If these signals could be harnessed to activate expression of therapeutic or protective gene products, the success of the stem cell-based therapy might be improved.

For example, when engrafted into a heart, stem cells that sense and respond to hypoxia by expressing a cardioprotective gene that promotes the long-term survival of the transplanted stem cells and surrounding tissue in the pathologic (hypoxic) environment would be useful for treating cardiac infarction or for preventing pathology due to cardiac ischemia.

Brief Summary of the Invention

The invention relates to the fields of biology, gene therapy, cell therapy and medicine. More particularly, the invention relates to compositions and methods for modifying a tissue by delivering to the tissue cells that have been engineered to express protective or therapeutic genes in response to a particular stimulus.

A system for modulating tissue physiology has been developed. The system employs genetically manipulated "vigilant cells", that are useful for improving the survival of such cells as well as other cells in a locally pathologic (*e.g.*, hypoxic) environment. Integrated within the genomes of vigilant cells are stable vectors containing a gene switch/biosensor and a gene amplification system to prevent or reverse tissue damage caused by disease. Grafted vigilant cells modified in this manner might more efficiently supplement the function of weakened regenerated tissue compared to non-vigilant cells (*i.e.*, cells lacking a gene switch/biosensor and a gene amplification system) because the vectors allow transgene (*e.g.*, a protective gene) expression in the regenerated tissue from vigilant cells to be regulated (*e.g.*, switched on or off) in response to a physiological signal, and to provide sufficient levels of the transgene product to effect a desired result, *e.g.*, prevention or reversal of cell damage. Multiple protective genes can be delivered simultaneously by the invention in response to a single stimulus to, for example, prevent or reverse tissue damage caused by disease.

While the system of the invention may be used in a number of different applications (as treatment or prophylaxis for diseases such as diabetes, cancer, stroke, pulmonary fibrosis, arthritis, atherosclerosis and inflammation), one application utilizes cells that include a dual rAAV vector system to detect and respond to hypoxia in cardiac tissue. The first rAAV vector in the system is the "sensor" vector. This vector contains a promoter linked to a sequence encoding an oxygen-sensitive chimeric transactivator, which is termed a redox sensor toggle (RST). The redox refers to reduced oxygen

availability and the toggle refers to the ability of the system to switch on genes. The RST contains a GAL4 DNA-binding domain (DBD), an oxygen-dependent degradation domain (ODD), and a p65 activation domain (p65 AD). The second rAAV vector is the "effector" vector which contains a cardioprotective gene linked to a GAL4 upstream activating sequence (UAS). The sensor rAAV vector expresses the chimeric transactivator specifically in the heart. In response to hypoxia, the transactivator binds to the GAL4 UAS sequence in the effector rAAV vector, resulting in the expression of the cardioprotective gene. Vigilant cells can thus be delivered to the myocardium as a way of preventing or reversing tissue damage associated with hypoxia.

10 The vigilant cells of the invention and methods of their use exhibit many advantageous characteristics. Importantly, the vigilant cell system is an on/off system that can be regulated by a signal related to a pathologic condition (e.g., hypoxia associated with ischemia). The system is also advantageous in that it can be used to treat or prevent a wide variety of different disease states. In addition, the system allows the use of naturally occurring transgenes which permits it to function in a way that more closely resembles the physiological condition than other systems and also reduces the chance that an adverse event (e.g. tumor formation) might occur. The vigilant cells can also be used to induce an anti-apoptotic effect in treated tissues.

Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Commonly understood definitions of molecular biology terms can be found in Rieger *et al.*, Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York, 1991; and Lewin, Genes V, Oxford University Press: New York, 1994. Commonly understood definitions of virology terms can be found in Granoff and Webster, Encyclopedia of Virology, 2nd edition, Academic Press: San Diego, CA, 1999; and Tidona and Darai, The Springer Index of Viruses, 1st edition, Springer-Verlag: New York, 2002. Commonly understood definitions of microbiology can be found in Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 3rd edition, John Wiley & Sons: New York, 2002.

30 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents and other

references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions will control. The particular embodiments discussed below are illustrative only and not intended to be limiting.

5

Brief Description of the Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 is a schematic depiction of two plasmid constructs used in the invention.

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Figures 2A-2C are photomicrographs showing *in vitro* human heme oxygenase-1 α (hHO-1) expression in vigilant cells under hypoxic (Figure 2A) versus normoxic (normal oxygen) conditions (Figure 2B), and a Western blot showing that total hHO-1 levels in hypoxia-treated MSCVHO-1 was on average 5.18-fold more abundant than at normoxia ($p < 0.01$).

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Figures 3A-3C are photomicrographs showing vigilant cells expressing hHO-1 in ischemic myocardium after cell transplantation. Surviving vigilant cells were identified by DAPI-labeled nuclei (Figure 3B), hHO-1 expression (TRITC) (Figure 3C), and both DAPI and TRITC staining (Figure 3A).

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Figures 4A-4C are photomicrographs showing vigilant cells expressing α -actin in ischemic myocardium after cell transplantation. Surviving vigilant cells were identified by DAPI-labeled nuclei (Figure 4B), α -actin expression (TRITC) (Figure 4C), and both DAPI and TRITC staining (Figure 4A). It was observed that groups of cells were positively stained for α -actin at injection sites in the peri-infarct area at 14 days after the transplantation of MSC_{VHO-1}, suggesting that some of the surviving MSCs had differentiated into myocyte-like cells within the native myocardium.

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Figures 5A-5C are photomicrographs showing vigilant cells expressing Connexin43 in ischemic myocardium after cell transplantation. Surviving vigilant cells were identified by DAPI-labeled nuclei (Figure 5B), connexin43 expression (TRITC) (Figure 5C), and both DAPI and TRITC staining (Figure 5A). It was observed that groups of cells were positively stained for connexin43 at injection sites in the peri-infarct area at 14 days after the transplantation of MSC_{VHO-1}, suggesting that some of the surviving MSCs had differentiated into myocyte-like cells within the native myocardium.

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Figures 6A-6B are confocal images comparing apoptosis (TUNEL assay) in a vigilant cells group versus a mesenchymal stem cells (MSCs) group in the peri-infarct zone of ischemic myocardium 1 week after transplantation. Vigilant cell group (Figure 6A); MSCs group (Figure 6B).

5 Figure 7 shows a schematic diagram of two plasmid constructs used in the invention which contain vigilant hHO-1 system and vigilant LacZ system, including sensor plasmid (top) and effector plasmid (bottom).

Figures 8A-8I are photomicrographs showing the effect of vigilant hHO-1 transfection on MSCs *in vitro*. To test the capability of MSC_{VHO-1} to resist ischemia/reperfusion damage, the present inventors used hypoxic/normoxic/hypoxic treatment on MSC_{VHO-1} with 24h hypoxia (1%O₂), 1 h normoxia (20%O₂) followed by another 24h hypoxia. Most of MSC_{VHO-1} expressed human HO-1 in immunocytostaining while negligible human HO-1 expression was seen in MSC_{VlacZ} or MSCs. The increase in human HO-1 expression in MSC_{VHO-1} was accompanied by a decrease in the MSCs apoptosis. As a result, the rate of cell apoptosis by the TUNEL in MSC_{VlacZ} or MSCs (5.06±0.95 and 5.32±1.03 positive nuclei per 500 cells respectively) exceed that of MSC_{VHO-1} (3.00±0.3 TUNEL+ per 500 cells) by 1.7-fold (p<0.01 for both MSC_{VlacZ} and MSCs). Figures 8A-8C: 4',6-diamidino-2'-phenylindole (DAPI); Figures 8D-F: TUNEL; and Figures 8G-8I: hHO-1.

20 Figure 9 shows a Western blot comparing expression level of the pro-apoptotic gene Bax in the cell lysate of MSC_{VHO-1} in comparison with MSC_{VlacZ} and MSCs.

Figures 10A-10F show the effect of vigilant hHO-1 transfection on MSCs in ischemic myocardium. In the MSC_{VHO-1} group, it was observed that a significantly smaller percentage of implanted cells were TUNEL positive (2.32±0.87 TUNEL+ per 200) (Figure 10D) compared with the MSC_{VlacZ} (4.98±0.90 TUNEL+ per 200, p<0.01 versus MSC_{VHO-1}) (Figure 10E), and MSCs group (5.54±0.95 TUNEL+, p<0.01 versus MSC_{VHO-1}) (Figure 10F). Figures 10-10C show cells of groups MSC_{VHO-1}, MSC_{VlacZ}, and MSCs, respectively, labeled with 4',6-diamidino-2'-phenylindole (DAPI).

Figures 11A-11I are micrographs showing immunofluorescent staining of hHO-1 in ischemic myocardium, demonstrating a higher level of hHO-1 expression in most of the implanted cells in the MSC_{VHO-1} group (Figure 11D), compared with the MSC_{VlacZ} (Figure 11E) and MSCs group (Figure 11F). Moreover, ischemic myocardium appeared

to express higher hHO-1 level in the MSC_{VHO-1} group than that of the medium group over the period studied. Figures 11A-11C show implanted cells of the MSC_{VHO-1} group, MSC_{VlacZ}, and MSC group, respectively, labeled with DAPI. Figures 11D-11F show hHO-1 staining in implanted cells of the MSC_{VHO-1} group, MSC_{VlacZ}, and MSC group, respectively. Figures 11G-11I: Merge.

Figures 12A-12D show photomicrographs demonstrating injection of MSCs inhibits left ventricular remodeling. Hearts injected with MSCs post-myocardial infarction and stained with Masson showed infiltration of island-like extension of organized cardiac tissue into the myocardial scar. Figure 12A: MSC_{VHO-1}; Figure 12B: MSC_{VlacZ}; Figure 12C: MSCs; and Figure 12D: medium.

Figures 13A and 13B are bar graphs indicating that the MSC_{VHO-1} group showed less infarct size than MSC groups in comparison with the medium group ($38.9 \pm 18.8\%$ versus $60.3 \pm 13.8\%$, $p=0.041$) (Figure 13B); and the infarct wall of left ventricular was thicker in MSC_{VHO-1} group in compared with MSC group ($1239.9 \pm 174.1 \mu\text{m}$ versus $979.1 \pm 85.6 \mu\text{m}$, $p=0.036$) (Figure 13A).

Figure 14 is a photomicrograph showing β -galactosidase staining of a section of whole heart injected with MSC_{VlacZ} (shown as a blue dot in the peri-infarct zone).

Figures 15A-15D show that implantation of MSCs improves left ventricular function. Both LV systolic performance (Figure 15A) and diastolic performance (Figure 15B), as assessed by maximum and minimum dP/dt respectively (Figures 15C and 15D, respectively), were greatest in the MSC_{VHO-1} group (maximum dP/dt 5717.1 ± 935.4 mmHg/s, minimum dP/dt -4893.3 ± 1435.1 mmHg/s), indicating that both systolic and diastolic functions were best preserved in the MSC_{VHO-1} group after myocardial infarction (maximum dP/dt, $P=0.11$ versus MSC_{VlacZ}, $P=0.002$ versus MSCs; minimum dP/dt, $P=0.01$ versus MSC_{VlacZ}, $P=0.006$ versus MSCs). These indicators were better in the MSC_{VlacZ} (maximum dP/dt 4336.0 ± 515.1 mmHg/s $P=0.14$ versus medium group, minimum dP/dt -3642.1 ± 667.0 mmHg/s $P=0.13$ versus medium group) and MSCs group (maximum dP/dt 3988.4 ± 450.1 mmHg/s, minimum dP/dt -3559.0 ± 713.0 mmHg/s $P=0.21$ versus medium group) than in the medium group (maximum dP/dt 3004.6 ± 362.7 mmHg/s, minimum dP/dt -2447.2 ± 621.9 mmHg/s), suggesting that MSCs implantation could preserve cardiac function after myocardial infarction.

Detailed Description of the Invention

The invention provides a system for modulating tissue physiology to, for example, prevent or reverse tissue damage caused by disease. The system utilizes cells that include stable vectors containing a gene switch/biosensor and a gene amplification system. The vectors allow transgene (*e.g.*, cardioprotective gene) expression in the vigilant cells to be regulated in response to a physiological signal, to be switched on or off, and to provide sufficient levels of the trans-gene product to effect a desired result, *e.g.*, prevention or reversal of cell damage. Multiple protective genes can be delivered simultaneously by the invention in response to a single stimulus. The vigilant cells can be delivered to a site of damaged tissue such as an infarct or to the site of tissue that might become damaged, *e.g.*, myocardium in a patient at high risk for a myocardial infarction (MI). The below described preferred embodiments illustrate adaptations of these compositions and methods. Nonetheless, from the description of these embodiments, other aspects of the invention can be made and/or practiced based on the description provided below.

15

Biological Methods. Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as *Molecular Cloning: A Laboratory Manual*, 3rd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and *Current Protocols in Molecular Biology*, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (*e.g.*, preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, *e.g.*, in *Current Protocols in Immunology*, ed. Coligan *et al.*, John Wiley & Sons, New York, 1991; and *Methods of Immunological Analysis*, ed. Masseyeff *et al.*, John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, *e.g.*, *Gene Therapy Methods*: ed. M.I. Phillips, Vol. 436, *Methods in Enzymology*, Academic Press, 2002; *Gene Therapy: Principles and Applications*, ed. T. Blackenstein, Springer Verlag, 1999; *Gene Therapy Protocols (Methods in Molecular Medicine)*, ed. P.D. Robbins, Humana

Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C.P. Hodgson, Springer Verlag, 1996.

Cells. Vectors containing a gene switch/biosensor and a gene amplification system might be introduced into a number of different cell types depending on the particular application of the invention. For example, cells (e.g., muscle cells such as cardiomyocytes, hepatocytes, tubular cells in kidney, type I and II pneumocytes in lung, ependymal cells and cells from the subventricular zone in central nervous system, blood cells such as B cells or T cells, and duct cells in pancreas, epidermal cells in skin, endothelial cells, fat cells, epithelial cells, neurocells, Schwann cells, and so forth) from a target tissue in an animal subject may be isolated from the subject, processed *ex vivo* to introduce the gene switch/biosensor and gene amplification system, and then delivered back to the target tissue in the subject. Because of their developmental plasticity, preferred cells for use in the invention are pluripotent or totipotent stem cells. Such stem cells may be embryonic (see, e.g., US patent numbers 5,843,780 and 6,200,806), neonatal, or from an adult animal. For transplantation into a subject, to avoid immune system-mediated rejection, cells of the invention are preferably histocompatible with the host subject.

For delivery to cardiac tissue, the use of hematopoietic stem cells and mesenchymal stem cells is preferred as these cells have been shown to differentiate into functional cardiomyocytes when in the presence of differentiated cardiomyocytes. See Orlic *et al.*, *Nature* 410:701-705, 2001; and Condorelli *et al.*, *Proc. Nat'l. Acad. Sci. USA* 98:10733-10738, 2001. MSC are particularly suited as a delivery vehicle for gene transfer. MSC can be obtained from a bone marrow aspirate taken from the iliac crest of adult animals (e.g., human subjects) and are well tolerated when transplanted to a host animal. MSC can be *ex vivo* expanded to large numbers and retain the ability to differentiate into cardiac, bone, adipocytes and muscle cells *in vitro* and *in vivo*. Mosca *et al.*, *Clin. Orthop.* 379 Suppl:S71-90, 2000; and Le Blanc, K., *Lakartidningen* 99:1318-1324, 2002. Muscle-derived stem cells (MDSCs) are also capable of delivering therapeutic genes and differentiating into a cardiomyocyte lineage within the heart. MDSCs are easily accessible via simple biopsy of the patient's own muscle. The isolation and characteristics of MDSCs and their use in regenerative medicine are reviewed in Sakai *et al.*, *Trends Cardiovasc. Med.* 12:115-120, 2002. Based on their differentiation

properties and facility of *ex vivo* expansion, human bone marrow mesenchymal progenitor cells (MPC) are also a suitable vehicle for delivering therapeutic genes to the bone marrow and other mesenchymal tissues. For example, MPC can be expanded *ex vivo* and infected with a rAAV virion of the invention or transduced with a stimulus-responsive rAAV vector(s). Conget *et al.*, *Exp. Hematol.* 28:382-390, 2000.

Cells genetically modified with the vectors of the invention can range in plasticity from totipotent or pluripotent stem cells (*e.g.*, adult or embryonic), precursor or progenitor cells, to highly specialized cells, such as those of the central nervous system (*e.g.*, neurons and glia), pancreas, heart, lung, and liver. Stem cells can be obtained from a variety of sources, including embryonic tissue, fetal tissue, adult tissue, umbilical cord blood, peripheral blood, bone marrow, and brain, for example. Methods and markers commonly used to identify stem cells and to characterize differentiated cell types are described in the scientific literature (*e.g.*, Stem Cells: Scientific Progress and Future Research Directions, Appendix E1-E5, report prepared by the National Institutes of Health, June, 2001). The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, umbilical cord blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

There are over 200 cell types in the human body, and the vectors of the subject invention can be introduced into any of them. A non-exhaustive list of cell types within into which vectors containing a gene switch/biosensor and a gene amplification system may be introduced is shown in Table 1. Other examples of cell types that can be genetically modified with the vectors of the invention include those disclosed by Spier R.E. *et al.*, eds., (2000) *The Encyclopedia of Cell Technology*, John Wiley & Sons, Inc., and Alberts B. *et al.*, eds., (1994) *Molecular Biology of the Cell*, 3rd ed., Garland Publishing, Inc., *e.g.*, pages 1188-1189.

Table 1. Examples of Target Cells	
Keratinizing Epithelial Cells	
keratinocyte of epidermis	
basal cell of epidermis	
keratinocyte of fingernails and toenails	
basal cell of nail bed	
hair shaft cells	

Table 1. Examples of Target Cells	
medullary	
cortical	
cuticular	
hair-root sheath cells	
cuticular	
of Huxley's layer	
of Henle's layer	
external	
hair matrix cell	
Cells of Wet Stratified Barrier Epithelia	
surface epithelial cell of stratified squamous epithelium of cornea tongue, oral cavity, esophagus, anal canal, distal urethra, vagina	
basal cell of these epithelia	
cell of urinary epithelium	
Epithelial Cells Specialized for Exocrine Secretion	
cells of salivary gland	
mucous cell	
serous cell	
cell of von Ebner's gland in tongue	
cell of mammary gland, secreting milk	
cell of lacrimal gland, secreting tears	
cell of ceruminous gland of ear, secreting wax	
cell of eccrine sweat gland, secreting glycoproteins	
cell of eccrine sweat gland, secreting small molecules	
cell of apocrine sweat gland	
cell of gland of Moll in eyelid	
cell of sebaceous gland, secreting lipid-rich sebum	
cell of Bowman's gland in nose	
cell of Brunner's gland in duodenum, secreting alkaline solution of mucus and enzymes	
cell of seminal vesicle, secreting components of seminal fluid, including fructose	
cell of prostate gland, secreting other components of seminal fluid	
cell of bulbourethral gland, secreting mucus	
cell of Bartholin's gland, secreting vaginal lubricant	
cell of gland of Littre, secreting mucus	
cell of endometrium of uterus, secreting mainly carbohydrates	
isolated goblet cell of respiratory and digestive tracts, secreting mucus	
mucous cell of lining of stomach	
zymogenic cell of gastric gland, secreting pepsinogen	
oxyntic cell of gastric gland, secreting HCl	
acinar cell of pancreas, secreting digestive enzymes and bicarbonate	
Paneth cell of small intestine, secreting lysozyme	
type II pneumocyte of lung, secreting surfactant	
Clara cell of lung	
Cells Specialized for Secretion of Hormones	
cells of anterior pituitary, secreting	

Table 1. Examples of Target Cells	
growth hormone	
follicle-stimulating hormone	
luteinizing hormone	
prolactin	
adrenocorticotrophic hormone	
thyroid-stimulating hormone	
cell of intermediate pituitary, secreting melanocyte-stimulating hormone	
cells of posterior pituitary, secreting	
oxytocin	
vasopressin	
cells of gut and respiratory tract, secreting	
serotonin	
endorphin	
somatostatin	
gastrin	
secretin	
cholecystokinin	
insulin	
glucagons	
bombesin	
cells of thyroid gland, secreting	
thyroid hormone	
calcitonin	
cells of parathyroid gland, secreting	
parathyroid hormone	
oxyphil cell	
cells of adrenal gland, secreting	
epinephrine	
norepinephrine	
steroid hormones	
mineralocorticoids	
glucocorticoids	
cells of gonads, secreting	
testosterone	
estrogen	
progesterone	
cells of juxtaglomerular apparatus of kidney	
juxtaglomerular cell	
macula densa cell	
peripolar cell	
mesangial cell	
Epithelial Absorptive Cells in Gut, Exocrine Glands, and Urogenital Tract	
brush border cell of intestine	
striated duct cell of exocrine glands	
gall bladder epithelial cell	
brush border cell of proximal tubule of kidney	

Table 1. Examples of Target Cells	
distal tubule cell of kidney	
nonciliated cell of ductulus efferens	
epididymal principal cell	
epididymal basal cell	
Cells Specialized for Metabolism and Storage	
hepatocyte	
fat cells (e.g., adipocyte)	
white fat	
brown fat	
lipocyte of liver	
Epithelial Cells Serving Primarily a Barrier Function, Lining the Lung, Gut, Exocrine Glands, and Urogenital Tract	
type I pneumocyte	
pancreatic duct cell	
nonstriated duct cell of sweat gland, salivary gland, mammary gland, etc.	
parietal cell of kidney glomerulus	
podocyte of kidney glomerulus	
cell of thin segment of loop of Henle	
collecting duct cell	
duct cell of seminal vesicle, prostate gland, etc.	
Epithelial Cells Lining Closed Internal Body Cavities	
vascular endothelial cells of blood vessels and lymphatics (e.g., microvascular cell)	
fenestrated	
continuous	
splenic	
synovial cell	
serosal cell	
squamous cell lining perilymphatic space of ear	
cells lining endolymphatic space of ear	
squamous cell	
columnar cells of endolymphatic sac	
with microvilli	
without microvilli	
"dark" cell	
vestibular membrane cell	
stria vascularis basal cell	
stria vascularis marginal cell	
cell of Claudius	
cell of Boettcher	
choroid plexus cell	
squamous cell of pia-arachnoid	
cells of ciliary epithelium of eye	
pigmented	
nonpigmented	
corneal "endothelial" cell	
Ciliated Cells with Propulsive Function	

Table 1. Examples of Target Cells	
of respiratory tract	
of oviduct and of endometrium of uterus	
of rete testis and ductulus efferens	
of central nervous system	
Cells Specialized for Secretion of Extracellular Matrix	
epithelial:	
ameloblast	
planum semilunatum cell of vestibular apparatus of ear	
interdental cell of organ of Corti	
nonepithelial:	
fibroblasts	
pericyte of blood capillary (Rouget cell)	
nucleus pulposus cell of intervertebral disc	
cementoblast/cementocyte	
odontoblast/odontocyte	
chondrocytes	
of hyaline cartilage	
of fibrocartilage	
of elastic cartilage	
osteoblast/osteocyte	
osteoprogenitor cell	
hyalocyte of vitreous body of eye	
stellate cell of perilymphatic space of ear	
Contractile Cells	
skeletal muscle cells	
red	
white	
intermediate	
muscle spindle-nuclear bag	
muscle spindle-nuclear chain	
satellite cell	
heart muscle cells	
ordinary	
nodal	
Purkinje fiber	
Cardiac valve tissue	
smooth muscle cells	
myoepithelial cells:	
of iris	
of exocrine glands	
Cells of Blood and Immune System	
red blood cell (erythrocyte)	
megakaryocyte	
macrophages	
monocyte	
connective tissue macrophage	

Table 1. Examples of Target Cells	
Langerhan's cell	
osteoclast	
dendritic cell	
microglial cell	
neutrophil	
eosinophil	
basophil	
mast cell	
plasma cell	
T lymphocyte	
helper T cell	
suppressor T cell	
killer T cell	
B lymphocyte	
IgM	
IgG	
IgA	
IgE	
killer cell	
stem cells and committed progenitors for the blood and immune system	
Sensory Transducers	
photoreceptors	
rod	
cones	
blue sensitive	
green sensitive	
red sensitive	
hearing	
inner hair cell of organ of Corti	
outer hair cell of organ of Corti	
acceleration and gravity	
type I hair cell of vestibular apparatus of ear	
type II hair cell of vestibular apparatus of ear	
taste	
type II taste bud cell	
smell	
olfactory neuron	
basal cell of olfactory epithelium	
blood pH	
carotid body cell	
type I	
type II	
touch	
Merkel cell of epidermis	
primary sensory neurons specialized for touch	
temperature	

Table 1. Examples of Target Cells	
primary sensory neurons specialized for temperature	
cold sensitive	
heat sensitive	
pain	
primary sensory neurons specialized for pain	
configurations and forces in musculoskeletal system	
proprioceptive primary sensory neurons	
Autonomic Neurons	
cholinergic	
adrenergic	
peptidergic	
Supporting Cells of Sense Organs and of Peripheral Neurons	
supporting cells of organ of Corti	
inner pillar cell	
outer pillar cell	
inner phalangeal cell	
outer phalangeal cell	
border cell	
Hensen cell	
supporting cell of vestibular apparatus	
supporting cell of taste bud	
supporting cell of olfactory epithelium	
Schwann cell	
satellite cell	
enteric glial cell	
Neurons and Glial Cells of Central Nervous System	
neurons	
glial cells	
astrocyte	
oligodendrocyte	
Lens Cells	
anterior lens epithelial cell	
lens fiber	
Pigment Cells	
melanocyte	
retinal pigmented epithelial cell	
iris pigment epithelial cell	
Germ Cells	
oogonium/oocyte	
spermatocyte	
Spermatogonium	
blast cells	
fertilized ovum	
Nurse Cells	
ovarian follicle cell	

Table 1. Examples of Target Cells
Sertoli cell
thymus epithelial cell (e.g., reticular cell)
placental cell

Introducing Exogenous Nucleic Acids into Host Cells. Cells of the invention include one or more nucleic acid constructs encoding a gene switch/biosensor and a gene amplification system. Use of such constructs to stably introduce exogenous nucleic acids into cells is generally known in the art and is described in methodology references such as Gene Therapy Methods: ed. M.I. Phillips, Academic press, 2002; Viral Vectors, eds. Yakov Gluzman and Stephen H. Hughes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988; Retroviruses, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000; Gene Therapy Protocols (Methods in Molecular Medicine), ed. Jeffrey R. Morgan, Humana Press, Totawa, NJ, 2001; and Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001.

Viral vectors are presently preferred for introducing a gene switch/biosensor and gene amplification system into cells. Viral vector methods and protocols are reviewed in Kay *et al. Nature Medicine* 7:33-40, 2001. Preferred viral vectors for use in the invention are recombinant AAV (rAAV) vectors. rAAV-mediated gene transfer is safe, stable and long-lasting. AAV-based vectors exhibit a high transduction efficiency of target cells and can integrate into the host genome in a site-specific manner. Methods for use of rAAV vectors are discussed, for example, in Tal, J., *J. Biomed. Sci.* 7:279-291, 2000 and Monahan and Samulski, *Gene Therapy* 7:24-30, 2000. As shown in Figure1, a first preferred rAAV vector in a gene switch/biosensor and gene amplification system (the sensor vector) contains a pair of AAV (inverted terminal repeats) ITRs which flank at least one cassette containing a promoter (e.g., a CMV promoter) which directs expression of an operably linked nucleotide sequence encoding a chimeric transactivator (e.g., GAL4DBD/ODD/p65AD). The second preferred rAAV vector (effector vector) contains a pair of AAV ITRs which flank several copies of a GAL4 UAS linked to a TATA element and therapeutic or protective gene (e.g., HO-1 α). Techniques involving nucleic acids and viruses of different AAV serotypes are known in the art and are described in Halbert *et al.*, *J. Virol.* 74:1524-1532, 2000; Halbert *et al.*, *J. Virol.* 75:6615-

6624, 2001; and Auricchio *et al.*, *Hum. Molec. Genet.* 10:3075-3081, 2001. The rAAV vectors and rAAV virions used in the invention may be derived from any of several AAV serotypes including 1, 2, 3, 4, 5, 6, 7, and 8. Preferred rAAV vectors for use in the invention are derived from serotype 2. Particular AAV vectors and AAV proteins of different serotypes are discussed in Chao *et al.*, *Mol. Ther.* 2:619-623, 2000; Davidson *et al.*, *Proc. Nat'l. Acad. Sci. USA* 97:3428-3432, 2000; and Xiao *et al.*, *J. Virol.* 72:2224-2232, 1998.

Other rAAV virions useful in the invention have mutations within the virion capsid. For example, rAAV mutants may have ligand insertion mutations for the facilitation of targeting rAAV virions to specific cell types (e.g., cardiac myocytes or pancreatic beta cells). The construction and characterization of rAAV capsid mutants including insertion mutants, alanine screening mutants, and epitope tag mutants is described in Wu *et al.*, *J. Virol.* 74:8635-45, 2000. Pseudotyped rAAV virions in which an rAAV vector derived from a particular serotype is encapsidated within a capsid containing proteins of another serotype may be used in methods of the invention (Halbert *et al.*, *J. Virol.* 74:1524-1532, 2000; Auricchio *et al.*, *Hum. Molec. Genet.* 10:3075-3081, 2001). Pseudotyped rAAV virions having mutations within the capsid may also be useful for introducing a gene switch/biosensor and gene amplification system to cells. Other rAAV virions that can be used in methods of the invention include those capsid hybrids that are generated by molecular breeding of viruses as well as by exon shuffling. See Soong *et al.*, *Nat. Genet.* 25:436-439, 2000; and Kolman and Stemmer *Nat. Biotechnol.* 19:423-428, 2001.

In addition to AAV, other viruses may be used to create vectors useful in preparing the vigilant cells of the invention. A list of such viruses include adenovirus (Ad) (see, W.C. Russell, *Journal of General Virology* 81:2573-2604, 2000, and Bramson *et al.*, *Curr. Opin. Biotechnol.* 6:590-595, 1995), Herpes Simplex Virus (see, Cotter and Robertson, *Curr. Opin. Mol. Ther.* 1:633-644, 1999), lentiviruses (see, Vigna and Naldini, *J. Gene Med.* 5:308-316, 2000 and Miyoshi *et al.*, *J. Virol.* 72:8150-8157, 1998), retroviruses (see Hu and Pathak, *Pharmacol. Rev.* 52:493-511, 2000 and Fong *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.* 17:1-60, 2000), and others (e.g., alphaviruses such as Semliki Forest Virus and Sindbis Virus).

Several non-viral methods for introducing a gene switch/biosensor and gene amplification system into host cells might also be used. For a review of non-viral methods, see Nishikawa and Huang, *Human Gene Ther.* 12:861-870, 2001 and M.I. Phillips, *Gene Therapy Methods*, Academic press, 2002. Various techniques employing plasmid DNA for the introduction of transactivator (e.g., GAL4 DBD/ODD/p65AD) and reporter sequences (e.g., several copies of a GAL4 UAS linked to a TATA element and therapeutic gene (e.g., cardioprotective gene) into cells are provided for according to the invention. Such techniques are generally known in the art and are described in references such as Ilan, Y., *Curr. Opin. Mol. Ther.* 1:116-120, 1999, Wolff, J.A., *Neuromuscular Disord.* 7:314-318, 1997 and Arztl, Z., *Fortbild Qualitatssich* 92:681-683, 1998.

In addition to virus-based methods, techniques involving physical introduction of a gene switch/biosensor and gene amplification system into a host cell that might be used include the particle bombardment method (see Yang *et al.*, *Mol. Med. Today* 2:476-481 1996 and Davidson *et al.*, *Rev. Wound Repair Regen.* 6:452-459, 2000), electroporation (see, Preat, V., *Ann. Pharm. Fr.* 59:239-244 2001), and cationic lipid-based methods (see, Felgner *et al.*, *Ann. N.Y. Acad. Sci.* 772:126-139, 1995 and Lasic and Templeton, *Adv. Drug Delivery Rev.* 20:221-266, 1996.)

The presence of exogenous nucleic acid constructs in cells can be monitored by conventional methods. For example, colonies of cells derived from rAAV vector-transduced cells can be assessed by PCR, flow cytometry and immunochemistry. The functional capacity of rAAV vector-transduced cells can be examined by quantifying clonogenic efficiency and proliferative capacity. Frey *et al.*, *Blood* 91:2781-2792, 1998. Following implantation of the transduced cells into the host, therapeutic gene expression can be analyzed by immunocytochemistry and RT-PCR of tissue samples. Condorelli *et al.*, *Proc. Nat'l. Acad. Sci. USA* 98:10733-10738, 2001. PCR techniques may be used to identify and locate implanted cells that carry a rAAV vector and/or therapeutic gene. See Hou *et al.*, *Proc. Nat'l. Acad. Sci. USA* 96:7294-7299, 1999.

Gene switch/biosensor and Gene Amplification System For Various Disease States. Cells incorporating the dual vector system described above can be adapted to a particular disease state by using an appropriate tissue-specific promoter, an appropriate stimulus-responsive element, and appropriate therapeutic genes for treating the particular disease state. For example, the cells of the invention can be used to treat diabetes type 1

in a patient. For treating diabetes type 1, cells having both 1) a sensor vector containing a glucose-sensitive element, for example, in addition to the GAL4 DBD and p65 AD sequences and 2) an effector vector containing a pre-pro-insulin gene(s), for example, linked to the UAS and E1b TATA elements, might be used. After delivery to a target tissue (*e.g.*, a pancreas or liver), the sensor vector in the cells detects elevated glucose levels and in response expresses a chimeric transactivator protein. The transactivator protein then binds to the effector vector and activates amplified expression of the pre-pro-insulin gene(s). When glucose levels are decreased the system switches off.

In another example, cells containing a gene switch/biosensor and gene amplification system can be used to treat cancer. Cells having a sensor vector that detects tumor markers and expresses a transactivator protein that activates anti-growth or anti-angiogenesis genes in the effector vector may be administered to the site of a tumor. A further example of a disease state to be treated using cells having a gene switch/biosensor and gene amplification system is stroke. Cells harboring a sensor vector responsive to hypoxia can express a transactivator protein that activates expression of the tPA gene in the effector vector. Such cells can be delivered to the brain of a subject at risk for stroke. To treat inflammatory diseases such as arthritis, pulmonary fibrosis and atherosclerosis, cells containing a sensor vector responsive to any of a number of atherosclerosis indicators of inflammation including cytokines, MCP-1, c-reactive protein or elevated triglyceride, oxidised LDL cholesterol, Lp(a), homocysteine, and fibrinogen levels, as well as decreased HDL levels and endothelial-derived nitric oxide production, can be constructed. A transactivator protein expressed in response to such an indicator activates expression of a therapeutic gene in the effector vector. Therapeutic genes for atherosclerosis include those that encode: 1) proteins with hypolipidemic activity, 2) proteins that act on the cholesteryl ester transfer protein and lipase systems, 3) cholesterol-removing proteins, 4) proteins with fibrinolytic activity, 5) proteins that provide low density lipoprotein receptor replacement, and/or 6) proteins that induce vascular protection and disobliteration of occlusions (Sirtori, C.R., *Pharmacol. Ther.* 67:433-47, 1995) 7) HO-1 and 8) LOX-1 antisense. Other disease states include anemia and renal diseases that involve reduced oxygen. In the case of anemia, the therapeutic gene would be erythropoietin.

Therapeutic products encoded by vectors of the subject invention may also be interfering RNA, which reduce expression of a target gene within a cell *in vitro* or *in vivo*. The target gene may be endogenous to the cell, or may be exogenous to the cell, such as a viral gene or parasite gene.

5 Therapeutic gene products that occur naturally a subject being treated are preferred over synthetic molecules or drugs because, for example, treating the subject with a naturally occurring gene product more accurately mimics the physiological state and reduces the likelihood that an adverse effect will occur (*e.g.*, development of a tumor).

10 Cardioprotective Genes. In preferred embodiments, cells of the invention include vectors that encode cardioprotective genes for protecting cells from ischemia in response to hypoxia. Several cardioprotective genes are known, including HO-1. This protein degrades the pro-oxidant heme and generates carbon monoxide and antioxidant bilirubin, conferring myocardial protection from ischemia/reperfusion injury. Franz *et al.*, *Circ. Res.* 73:629-638, 1993. Another example of a cardioprotective gene is superoxide dismutase, which protects heart tissue from super oxide radicals generated during ischemia-reperfusion. Chen *et al.*, *Circulation* 94:II412-II417, 1996; and Woo *et al.*, *Circulation* 98:II255-II260, 1998. Genes that provide a protective effect from other cardiac disease states, such as heart degeneration and failure, may also be used in vectors
15 of the invention. An example of a gene that improves cardiac function is phospholamban (PLN). The PLN gene product regulates the strength of each heartbeat and is known to malfunction in heart failure. Zvaritch *et al.*, *J. Biol. Chem.* 275:14985-14991, 2000. Any suitable cardioprotective gene that provides a therapeutically effective level of protection may be used in the invention.

25 A number of antisense molecules that confer a cardioprotective effect are known, including antisense to angiotensin II type-1 receptor (Yang *et al.*, *Circulation* 96:922-926, 1997; and Yang *et al.*, *Circ. Res.* 83:552-559, 1998), antisense to adrenergic beta-1 receptor (Chen *et al.*, *Pharmacol. Exp. Ther.* 294:722-727, 2000), and antisense to angiotensin-converting enzyme that has been shown to protect rat hearts from ischemia-reperfusion (Chen *et al.*, *Pharmacol. Exp. Ther.* 294:722-727, 2000).
30

Delivery of Cells to a Target Tissue. Delivery of vigilant cells to a target tissue in an animal subject is provided for within the invention. Any suitable method might be

used (*e.g.*, directly to the site through epicardial puncture or endoventricular injection, intracoronary injection with angioplasty, pericardial injection, transdermal injection, or systemic infusion).

5 The cells described above are preferably administered to a mammal in an effective amount, that is, an amount capable of producing a desirable result in a treated subject (*e.g.*, protecting tissue from ischemia-mediated damage in the subject). As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, time and route of administration, general health, and drugs being
10 administered concurrently. It is expected that an appropriate dosage of vigilant cells for delivery to a human subject would be in the range of about 10^6 to 10^{10} cells. However, varying doses within the scope of the present invention will depend on the physiologic condition, size and weight of the subject and are evident to those of ordinary skill in the art.

15 As used in this specification, including the appended claims, the singular "a", "an", and "the" include plural reference unless the context dictates otherwise. Thus, for example, a reference to "a polynucleotide" includes more than one such polynucleotide. A reference to "a nucleic acid sequence" includes more than one such sequence. A reference to "a cell" includes more than one such cell.

20 The terms "comprising", "consisting of" and "consisting essentially of" are defined according to their standard meaning. The terms may be substituted for one another throughout the instant application in order to attach the specific meaning associated with each term.

The term "polypeptide" refers to any polymer comprising any number of amino
25 acids, and is interchangeable with "protein", "gene product", and "peptide".

A nucleic acid molecule can be isolated from a natural source, or it can be produced using recombinant DNA technology (*e.g.*, polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid molecules can be generated or modified using a variety of techniques including, but not limited to, classic
30 mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments,

polymerase chain reaction (PCR) amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to “build” a mixture of nucleic acid molecules, and combinations thereof.

Although the phrase “nucleic acid molecule” (or “nucleotide molecule”) primarily refers to the physical nucleic acid molecule and the phrase “nucleic acid sequence” primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably. As used herein, a “coding” nucleic acid sequence refers to a nucleic acid sequence that encodes at least a portion of a polypeptide (*e.g.*, a portion of an open reading frame), and can more particularly refer to a nucleic acid sequence encoding a polypeptide which, when operatively linked to a transcription control sequence (*e.g.*, a promoter sequence), can express the polypeptide.

The term “operably-linked” or “operatively linked” is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably-linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence. For example, a coding sequence is operably-linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence, and the promoter sequence can still be considered “operably-linked” to the coding sequence.

As used herein, the term “isolated” means removal from its native environment, and can include removal from its immediate native environment.

As used herein, the term “differentiated” refers to those cells that maintain in culture all, or a substantial amount of, their specialized structure and function typical of the cell type *in vivo*. Partially differentiated cells maintain less than a substantial amount of their full complement of specialized structure and/or function.

Nucleic acid constructs comprising a gene switch/biosensor and a gene amplification system may be introduced into stem cells. As used herein, the term “stem cell” is an unspecialized cell that is capable of replicating or self renewal, and developing into specialized cells of a variety of cell types. The product of a stem cell undergoing

division is at least one additional stem cell that has the same capabilities of the originating cell. For example, under appropriate conditions, a hematopoietic stem cell can produce a second generation stem cell and a neuron. Stem cells include embryonic stem cells (*e.g.*, those stem cells originating from the inner cells mass of the blastocyst) and adult stem cells (which can be found throughout the more mature animal, including humans). As used herein, stem cells are intended to include those stem cells found in animals that have matured beyond the embryonic stage (*e.g.*, fetus, infant, adolescent, juvenile, adult, *etc.*). The list of tissues reported to contain stem cells is growing and includes, for example, bone marrow, peripheral blood, brain, spinal cord, umbilical cord blood, amniotic fluid, placenta, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

Nucleic acid constructs comprising a gene switch/biosensor and a gene amplification system may be introduced into progenitor cells. As used herein, the term “progenitor cell” (also known as a precursor cell) is unspecialized or has partial characteristics of a specialized cell that is capable of undergoing cell division and yielding two specialized cells. For example, a myeloid progenitor/precursor cell can undergo cell division to yield two specialized cells (a neutrophil and a red blood cell).

As used herein, the term “phenotype” refers to all the observable characteristics of a cell (or organism); its shape (morphology); interactions with other cells and the non-cellular environment (*e.g.*, extracellular matrix); proteins that appear on the cell surface (surface markers); and the cell’s behavior (*e.g.*, secretion, contraction, synaptic transmission).

As used herein, the terms “administer”, “apply”, “treat”, “transplant”, “implant”, “deliver”, and grammatical variations thereof, are used interchangeably to provide vectors of the subject invention to target cells *in vitro* (*e.g.*, *ex vivo*) or *in vivo*, or provide genetically modified cells of the subject invention to a subject.

As used herein, the term “co-administration” and variations thereof refers to the administration of two or more agents simultaneously (in one or more preparations), or consecutively. For example, one or more types of genetically modified cells of the invention can be co-administered with other agents.

Examples

The invention can be illustrated by the following examples in which bone marrow- derived stem cells that incorporate a dual viral vector system for expressing cardio-protective genes are administered to infarcted heart tissue to protect cells from ischemia-induced damage.

Materials and Methods for Example 1

Culture of mouse mesenchymal stem cells (MSCs). Four to six-week-old male BALB/c mice obtained from Harlan Company were killed by an overdose of pentobarbital injected intraperitoneally. The mice were immersed in 70% alcohol for 8 minutes, after which time the tibia and femoral bones were excised and collected. Bone marrow was obtained by flushing the shaft of femur and tibia using a 27g needle attached to a 3ml syringe with Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (100U/ml penicillin G, 100ug/mg streptomycin). The marrow was disaggregated by gently aspirating several times using the same needle and syringe. The cells thus obtained were centrifuged for 5 minutes at 2000 rpm. The resulting cell pellet was resuspended and the cell concentration was adjusted to 5×10^7 nucleated cells per ml in complete medium (DMEM supplemented with 20% fetal bovine serum (FBS) and 100U/ml penicillin G and 100ug/mg streptomycin). The cells were then placed into a 75cm² Corning flask and cultured in a humidified incubator at 37°C with 5% CO₂.

MSC Isolation and Expansion. In the cultures, the medium was replaced every 4 days, keeping the adherent cells and discarding nonadherent cells. Each primary culture was passaged to two new flasks when the MSCs grew to approximately 70% confluence. After a series of passages, homogeneous MSCs that were devoid of hematopoietic cells resulted. These were used for cell transfection.

Vector Construction. As shown in Figure1, a double plasmid system was developed that includes a sensor plasmid (pGS-ODD) derived from pGS-CMV, which expresses a chimeric transcription factor consisting of the yeast GAL4 DBD (DBD amino acids 1-93) and the human p65 AD (AD amino acids 283-551) from NF- κ B under the control of a CMV enhancer/promoter. An ODD (amino acids 394-603 within HIF-1 α) was amplified by PCR from pCEP4/HIF-1 α and fused in frame between the coding

sequences of GAL4DBD and p65 AD to generate pGS-ODD. The second component of the system is an effector plasmid (pGene-hHO-1) that was generated by replacing the LacZ gene in pGene-LacZ (INVITROGEN, Carlsbad, CA) with HO-1 α cDNA. In the construct the HO-1 α insert was driven by six copies of a 17bp GAL4 UAS and an
5 adenovirus E1b TATA box.

The expression cassette of pGS-ODD was cloned into an AAV vector between two ITRs to generate the prAAV-GS-ODD. In the same way, the expression cassette of pGene-hHO-1 (GAL4UAS, E1bTATA box, human HO-1 α and 6xHis) was cloned into an AAV backbone that contains green fluorescent protein (GFP) to generate pAAV-hHO-1.
10 This rAAV vector plasmid and helper plasmid pDG were cotransfected into 293 cells by the calcium phosphate precipitation method. Plasmid pDG carries the rAAV rep and cap genes, as well as Ad helper genes required for rAAV replication and packaging. The CaPO₄/DNA precipitate was incubated in the media at 37°C and 5% CO₂ for about 48 hrs. After harvesting the cells, the produced virions were isolated by (1) placing the cells in
15 lysis buffer, (2) performing three cycles of freezing and thawing the cells for three times and (3) low speed centrifugation to remove cellular debris. The rAAV in the resulting supernatant was further purified using a non-ionic iodixanol gradient separation followed by heparin affinity chromatography. The method results in more than 50% recovery of rAAV from a crude lysate and routinely produces virus that is more than 99% pure with
20 particle-infectivity ratios of less than 100. The titering of rAAV stock was performed by quantitative competitive PCR (QC-PCR) and fluorescent cell assay.

Genetic Modification. At a confluence of 50%~60%, MSCs were treated with 0.25% trypsin and 1mM EDTA (Gibco, Carlsbad, CA) and replated. The next day, cells were infected with two viruses (AAV-MLC-ODD & AAV-hHO-1-6xHis) using
25 LipofectAMINE (INVITROGEN, Carlsbad, CA) according to manufacturer's protocol. Three days after the transduction, MSCs were put into hypoxia chambers (Oxygen Sensors) with 1% O₂, 5% CO₂, and 94%N₂, and then incubated at 37°C. After 5 hours, the cells were screened for GFP expression using an inverted microscope with appropriate filters. The GFP-positive clones were recognized and cloned to establish stable cell lines
30 that express the reporter gene under hypoxia constitutively.

Cloning of Vigilant cell. Medium in the culture dish was removed and the dish was washed twice with phosphate-buffered saline (PBS) to remove any trace of serum. A

cloning ring was placed over a selected green colony under a fluorescence microscope after hypoxia treatment. 75 μ l of 0.25% trypsin 1mM EDTA solution (GIBCO, Carlsbad, CA) was added to the center of the ring with a pipette. The dish was returned to the incubator for 15 min, after which time the colony was examined under a microscope to ensure the cells rounded up and detached. The area in the ring was pipetted to detach all cells, which were then transferred to centrifuge tube. Serum-containing medium was added to neutralize the trypsin. The cells were centrifuged and resuspended in culture medium and placed in culture. This procedure was repeated until stable hypoxia-responding MSCs clones were obtained. These were named "vigilant cells."

10 Vigilant cell expansion and labeling. Purified vigilant cells were further expanded in culture until the day of implantation. Prior to implantation, MSCs were stained with the fluorescent DNA-intercalating dye 4, 6-diamidino-2-phenylindole (DAPI). Prior to injection the cells were thoroughly washed and kept at 4°C.

Myocardial Infarction (MI). Mice (6 to 8-week-old male BALB/c) were anesthetized with sodium pentobarbital (40mg/kg, i.p.) and then intubated with a 24-gauge intravenous catheter with a tapered tip via the oral cavity without tracheotomy. Mice were mechanically ventilated on a Harvard rodent ventilator (Harvard Apparatus Co. Inc, Boston, MA) with room air (respiratory rate 110/min, tide volume: 0.3ml, ventilatory rate of 110/min was found to produce optimal values of pO₂, pCO₂, and pH).
20 Under the dissecting microscope, the chest was opened through a left thoracotomy in the fourth intercostal space using a microcoagulator. The medial aspect of the incision was extended cranically to form a flap that was retracted to expose the heart. A small opening was made in the pericardium and an 8-0 nylon suture was passed under the left anterior descending coronary artery 2-3mm from the tip of the left auricle. Coronary occlusion
25 was induced by ligating the suture. Successful performance of coronary occlusion was verified by visual inspection (noting the development of a pale color in the distal myocardium after ligation). After the coronary occlusion protocol was completed, the chest was closed in layers by a 5-0 polyester fiber suture with one layer through the chest wall. The muscle was closed with a polypropylene suture and the skin was closed with a
30 stainless steel wound clip (removed 10 days post-operation). The animal subjects were removed from the respirator and kept warm by a heated water blanket and allowed 100% oxygen via nasal cone.

Vigilant cell implantation. One week after MI induction, 10 mice were injected with DAPI-labeled vigilant cells (6×10^6 in $50 \mu\text{l}$ of DMEM medium), and 10 mice were injected with DAPI-labeled MSCs as follows. Anesthesia was induced and maintained. Mice were intubated and ventilated. The heart was re-exposed via formal left thoracotomy incision. Under direct vision, the cell suspension was injected into the peri-infarct region with a 31-gauge needle. The chest was closed in layers and the mice were sacrificed at 7 days after cell therapy.

Immunohistochemistry on heart tissue. The survival of implanted MSCs was examined in mice by measuring the DAPI positive cells in sections harvested from ischemic myocardium as follows. Mice in both groups were sacrificed at 1 week after cell implantation. Heart sections harvested from the left ventricle were embedded in Tissue-Tek O.C.T. compound (Miles Inc, Elkhart, IN), then snap-frozen in dry ice. DAPI-positive MSC nuclei were quantified in frozen sections by fluorescence microscopy (Zeiss Axioplan 2 microscope). The survival of vigilant cells was compared to that of control MSCs. Heart sections were selected by immunofluorescent staining for hHO-1 as follows. Sections were incubated in primary antibodies, the mouse anti-human heme oxygenase 1 IgG₁ (BD Transduction Laboratories, San Jose, CA), at a 1:50 dilution for 30 minutes at 37°C , and then incubated with secondary antibodies, goat anti-mouse IgG-TRITC conjugate (SIGMA, Saint Louis, MO), at a 1:200 dilution for 30 minutes in 37°C .

Regeneration of myocardial tissue. The following cardiac markers were tested using primary mouse monoclonal antibodies to the following: α -actin (sarcomeric) clone EA-53 (SIGMA, Saint Louis, MO); connexin-43 (BD Transduction Laboratories, San Jose, CA) and desmin (BD Transduction Laboratories). Sections were incubated in the respective primary antibodies at a 1:50 dilution for 30 minutes at 37°C , and then incubated in second antibody, goat anti-mouse IgG-TRITC conjugate (SIGMA, Saint Louis, MI), at a 1:200 dilution for 30 minutes in 37°C . Combined DAPI and TRITC images were made by using a simultaneous excitation filter under fluorescence microscopy (Zeiss Axioplan 2 microscope). Digital images were transferred to a computer equipped with Spot Software (Diagnostic Instruments, Inc., Sterling Heights, MI) for analysis.

Apoptosis. Five μm cryosections cut from the midcavity of mouse heart were stained with fluorescent TUNEL using a commercially available kit (TUNEL Apoptosis Detection Kit upstate, Lake Placid, NY) according to the manufacturer's instructions. In brief, thin myocardial sections were treated with proteinase K (250 μl); incubated with biotin-dUTP, TdT; and then treated with avidin-FITC. Apoptotic cells were visualized and acquired using a BIO-RAD 1024 ES Confocal Microscope. The number of TUNEL-positive cells was scored in peri-infarct regions. For each heart, the number of TUNEL-positive myocyte nuclei was scored per unit field. Images were taken at high magnification using 100X.

Example 1—Results

In vitro hHO-1 expression in vigilant cells under hypoxic conditions (Figure 2A, with 1% O_2) was compared to the same cells under normoxic conditions (Figure 2B, 20% O_2). MSCs were transfected with pGS-ODD and pGene-hHO-1 plasmids. The immunofluorescence staining with an antibody to hHO-1 revealed intense expression in the cells placed in hypoxic conditions. (X 100, BIO-RAD Confocal microscope, Hercules, CA), but not in the cells placed in normoxic conditions.

Human HO-1 expression by vigilant cells in ischemic myocardium was examined after cell transplantation. Immunofluorescence staining for hHO-1 revealed expression of hHO-1 in grafted vigilant cells in the peri-infarct zone. Surviving vigilant cells were identified by DAPI-labeled nuclei as shown in Figure 3B. Expression of hHO-1 (TRITC immunofluorescence) is shown in Figure 3C. Figure 3A shows both DAPI and TRITC staining. Most of the surviving vigilant cells stained with DAPI. Many of the cells in the transplant stained positively for hHO-1.

Connexin43 expression by vigilant cells in ischemic myocardium was examined after cell transplantation. Immunofluorescence staining for Connexin43 revealed that grafted Connexin43-expressing vigilant cells localized mainly in sites parallel to host myocardium. Surviving vigilant cells were identified by DAPI labeled nuclei as shown in Figure 5B. Expression of Connexin43 (TRITC immunofluorescence) is shown in Figure 5C. Figure 5A shows both DAPI and TRITC staining. A portion of the vigilant cells in the transplant stained positively for Connexin43, an intercalated disk structure protein.

As shown in Figures 4A-4C, α -actin expression by vigilant cells in ischemic myocardium was examined after cell transplantation. Immunofluorescence staining for α -actin revealed expression of α -actin in grafted vigilant cells in the peri-infarct zone. Surviving vigilant cells were identified by DAPI-labeled nuclei as shown in Figure 4B. Expression of α -actin (TRITC immunofluorescence) is shown in Figure 4C. Figure 4A shows both DAPI and TRITC staining. A portion of the vigilant cells in the transplant also stained positively for α -actin.

As shown in Figures 6A and 6B, apoptosis in the vigilant cells group versus MSCs in ischemic myocardium 1 week after transplantation was examined by TUNEL assay. Fewer apoptotic nuclei were observed in the vigilant cell group (Figure 6A) than in the MSCs group (Figure 6B) in the peri-infarct zone.

Materials and Methods for Examples 2-5

Animals. All studies were performed with the approval of the institutional ethics committee. The investigation conformed to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Both donors and recipients were male BALB/c inbred mice weighing 20 to 25 g.

Cell Isolation, Culture, and Labeling. Bone marrow was flushed from tibias and femurs using a 25g needle. Whole marrow cells were cultured at $1 \times 10^6/\text{cm}^2$ in MesenCult basal medium supplemented with mesenchymal stem cell stimulatory supplements (StemCell Technologies Inc, Vancouver, Canada). The nonadherent cells were removed by a medium change at 72 hours and every 4 days thereafter. The monolayer, referred to as MSCs, was expanded by two passages. Before implantation, MSCs were trypsinized, washed, and labeled with 4',6-diamidino-2'-phenylindole (DAPI, SIGMA-Aldrich Co, St. Louis, MO), as previously described. After labeling, DAPI stains specifically 100% of the MSCs nuclei.

Gene Construction and Gene Transfection. The vigilant plasmid system is a double-plasmid system, which contains a sensor plasmid with the oxygen sensitive toggle (OST) and effector plasmid with protective gene. In the sensor plasmid, (pS-CMV-OST), the oxygen-dependent degradation domain (ODD, amino acids 394-603) was amplified by PCR from pCEP4/HIF-1 α and inserted in the frame between the coding sequence of

GAL4DNA binding domain and p65 activation domain in pS-CMV to generate pS-CMV-OST (Figure 7). The effector plasmid (pEhHO-1) (Figure 7) was constructed by replacing the LacZ coding sequence with full-length cDNA for human heme oxygenase-1 α in pE/V5-His/LacZ (INVITROGEN Corporation, Ca). A six-copy His tag was added to the C-terminal of hHO-1. Human HO-1 is driven by six copies of a 17-bp GAL4 UAS and an adenovirus-derived E1b TATA box. The construction of the plasmids was confirmed by nucleotide sequence analysis.

Polyethylenimine-Transfection Kit (Tf PEI-Kit, Bender MedSystems, Vienna, Austria) was prepared as described previously. Briefly, 2 plasmids (1:1) was mixed with a Tf PEI to generate a Tf PEI/DNA complex suspension. MSCs at 70% confluence were incubated with the Tf PEI/DNA containing the sensor plasmid and effector plasmid for 4 hours at 37°C. Control cells were transfected with vigilant vector with LacZ as reporter using the same protocol.

In Vitro Hypoxia treatment and apoptosis Assessment. To evaluate hypoxia regulation of vigilant hHO-1 vectors *in vitro*, cell transfection and hypoxic treatment was performed as performed as described previously. MSCs was transfected with 1 μ g pS-CMV-OST and 0.5 μ g pE-hHO-1. Twenty-four hours after transfection, the medium was changed and MSCs were incubated at 1% or 20% O₂ for 24 hours before preparation of lysates. Each condition was run in triplicate.

To test the capability of resistance ischemia/reperfusion damage of MSC_{VHO-1}, the present inventors used hypoxic/normoxic/hypoxic treatment on MSC_{VHO-1} with 24h hypoxia (1%O₂), 1 hour normoxia (20%O₂) followed by another 24h hypoxia. Western blot analysis was performed using 10-15 μ g of whole cell. The hHO-1/6xHis was probed with monoclonal anti-6xHis antibody (INVITROGEN, Carlsbad, Ca) and monoclonal anti-hHO-1 antibody (BD Biosciences, Palo Alto, Ca). The pro-apoptosis protein Bax was probe with monoclonal anti-Bax antibody (Upstate, NJ). The internal control protein GAPDH antibody (Chemicon, Temecula, CA) was probed for cells. The antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ). For immunocytochemical analysis, MSCs were seeded into glass bottom microwell dishes (MatTek Corp, Ashland, MA) at day 2 after gene transfection for hypoxic treatment. The next day, samples were fixed with ice-cold ethanol. Half samples were incubated in a 1:50 dilution of anti-human HO-1 antibody (Santa Cruz

Biotechnology) at 37°C for 30min, followed by incubation in a 1:500 dilution of FITCconjugated secondary antibody (SIGMA). The other samples were undergone TUNEL assay. Nuclei were counterstained with 10 µg/mL DAPI.

Myocardial Infarction Model and Cell Implantation. Male BALB/c mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and mechanically ventilated. After the heart was exposed through a lateral thoracotomy, a 8-0 polypropylene thread was passed around the left coronary artery and the artery was occluded. At day 3 after gene transfection, MSCs were harvested using trypsin and resuspended in serum-free DMEM just before grafting to the heart. 1x10⁶ of vigilant hHO-1- transfected-MSCs (MSC_{VHO-1} group), vigilant lacZ-transfected MSCs (MSC_{VlacZ} group), MSCs (MSCs group) or medium (medium group) in 50µl volume was injected into syngenetic adult BALB/c mouse hearts in the border zone surrounding the infarct 1 hour after induction of myocardial infarction (injections of 1x10⁶ cells in 50 µL) with a 30G needle. The surgical wounds were repaired, and the mice were returned to their cages to recover. Aseptic surgical techniques were used throughout.

hHO-1 expression and differentiation of grafted cells in ischemia myocardium. At 14 days after cell transplantation, 6 hearts from each group were collected to assess the hHO-1 expression of grafted MSCs. The frozen left ventricular samples were cut into 6-µm sections and fixed with -20°C methanol. The sections were incubated with 0.6% H₂O₂, and immersed in 0.1% Triton X-100. After blocking with 1% bovine serum albumin, the sections were incubated with a 1:40 dilution of anti-HO-1 antibody (BD), anti-α-actin. This was followed by incubation with a 1:100 dilution of TRITC secondary antibody (SIGMA).

Functional Assessment. At 14 days after cell transplantation, mice from 3 groups (n=6 in each group) were anticoagulated by an intravenous injection of heparin. Mice were anesthetized by pentobarbital (40 mg per kg (body weight), intraperitoneally (i.p.)). Mikro-Tip pressure catheter transducers (Model SPR-671, Millar Instruments, Inc, Houston, TX) were cannulated into the LV chamber through right carotid artery. The left ventricular pressure was digitized using the commercially available data acquisition system (PowerLab/8sp, ADInstruments, Inc.). After steady state had been established, LV systolic pressure (LVSP), LV develop pressures (LVDP), and maximal rates of pressure rise and fall (±dP/dt) were recorded in the closed-chest preparation.

Infarct Size and myocardial apoptosis. After perfusion, the left ventricle was sectioned into 5 segments parallel to the apex-base axis and frozen in an embedding medium. A 10- μ m section was cut from each segment, fixed in 3.7% formaldehyde, and stained with Masson trichrome. Sections from all slices were projected onto a screen for computer-assisted planimetry. The ratio of scar length to left ventricular circumferences of the endocardium and epicardium was expressed as a percentage to define infarct size, Average thick of infarct wall was also detected by planimetry.

Statistical Analysis. All values are expressed as mean \pm SEM. The differences in the data between 2 groups were determined with a Student's t test. Statistical comparison of the data was performed with one-way ANOVA followed by LSD post-hoc testing. P<0.05 was considered significant.

Example 2—Human HO-1 expression protects against MSC apoptosis *in vitro*

Several groups have reported the use of bone marrow-derived mesenchymal stem cells (MSCs) for restoration of cardiac function. However, progress in cell therapy has been hampered by poor viability of implanted cells and the vulnerability of regenerated tissue to repeated bouts of ischemia. To assess the hypothesis that genetically modified MSCs can increase the survival rate of implanted MSCs and afford self-protection of regenerated cardiomyocytes, the present inventors transfected MSCs with a vigilant plasmid system that expresses human heme oxygenase (hHO-1 α) in response to hypoxia.

The MSCs isolated from the bone marrow of male BALB/C mice were grown to confluence and transfected with vigilant hHO-1, vigilant lacZ vector using polyethylenimine transferinfection system. 80% of transfected MSCs were positively stained for human HO-1 after hypoxia treatment (1% O₂ for 24 hours). Immunocyto staining for human HO-1 demonstrated stronger hHO-1 expression in hypoxia treated MSC_{VHO-1} compared to normoxia (Figures 2A and 2B). Western blot showed that total hHO-1 levels in hypoxia-treated MSCVHO-1 was on average 5.18-fold more abundant than at normoxia (P<0.01) (Figure 2C). Because there is a fusion gene hHO-1/6His in the vector, the expressed fusion protein reacts only with antibody to human HO-1 or 6xhis but not with endogenous mouse HO-1, these positively stained cells were presumed to be of vigilant hHO-1 origin in response to hypoxia, endogenous mouse HO-1 had no effect on the detection of vigilant HO-1 expression.

To test the capability of MSC_{VHO-1} to resist ischemia/reperfusion damage, the present inventors used hypoxic/normoxic/hypoxic treatment on MSC_{VHO-1} with 24 hour hypoxia (1%O₂), 1 hour normoxia (20%O₂) followed by another 24h hypoxia. Most of MSC_{VHO-1} expressed human HO-1 in immunocytostaining while negligible human HO-1 expression was seen in MSC_{VlacZ} or MSCs (Figures 8A-8I). The increase in human HO-1 expression in MSC_{VHO-1} was accompanied by a decrease in the MSCs apoptosis. As a result, the rate of cell apoptosis by the TUNEL in MSC_{VlacZ} or MSCs (5.06±0.95 and 5.32±1.03 positive nuclei per 500 cells respectively) exceed that of MSC_{VHO-1} (3.00±0.3 TUNEL+ per 500 cells) by 1.7-fold (p<0.01 for both MSC_{VlacZ} and MSCs) (Figures 8A-8I). Similarly, a down-regulation in a pro-apoptotic gene Bax level in the cell lysate of MSC_{VHO-1} was confirmed by Western blot in comparison with MSC_{VlacZ} and MSCs (Figure 9).

Example 3—Induction of human HO-1 expression in ischemic myocardium protect against MSC apoptosis *in vivo*

At 1 hour after myocardial infarction, the border zones of infarcts were injected with 1x 10⁶ MSC_{VHO-1}, MSC_{VlacZ}, MSCs or serum-free DMEM medium. At 12 days after injection, Apoptotic implanted cells were assessed in ischemic myocardium by TUNEL assay. In the MSC_{VHO-1} group, a significantly smaller percentage of implanted cells were TUNEL positive (2.32±0.87 TUNEL+ per 200) compared with the MSC_{VlacZ} (4.98±0.90 TUNEL+ per 200, p<0.01 versus MSC_{VHO-1}) and MSCs group (5.54 ±0.95 TUNEL+, p<0.01 versus MSC_{VHO-1}) (Figures 10A-10F). This finding is inversely correlated with immunofluorescent staining of hHO-1 in ischemic myocardium which demonstrated that higher level of hHO-1 expression in most of implanted cells in MSC_{VHO-1} group, compared with the MSC_{VlacZ} and MSCs group (Figures 11A-11I). Moreover, ischemic myocardium appeared to express higher hHO-1 level in the MSC_{VHO-1} group than that of the medium group over the period studied.

Example 4—In site differentiation of Grafted MSCs

Masson trichrome staining of post-myocardial infarction hearts injected with MSCs showed infiltration of island-like extension of organized cardiac tissue (Figures 12A-12D) into the myocardial scar. β -Galactosidase staining of section of whole heart

injected with MSC_{vlacZ} show blue dot in the peri-infarct zone (Figure 14). In ischemic hearts injected with MSC_{vHO-1}, groups of cells were positively stained for α -actin and connexin43 at injection sites in the peri-infarct area at 14 days after the transplantation of MSC_{vHO-1} (Figures 4A-4C and Figures 5A-5C). This suggested that some surviving MSCs had differentiated into myocyte-like cells within the native myocardium. Positive staining was also observed in the MSC_{vlacZ} groups and MSCs groups.

Example 5—Left ventricular remodeling and Cardiac Function after MSCs Transplantation

At day 14 after cell transplantation, MSC_{vHO-1} group showed less infarct size than MSC groups in comparison with the medium group ($38.9 \pm 18.8\%$ versus $60.3 \pm 13.8\%$, $p=0.041$). Further, infarct wall of left ventricular was thicker in MSC_{vHO-1} group in compared with MSC group ($1239.9 \pm 174.1 \mu\text{m}$ versus $979.1 \pm 85.6 \mu\text{m}$, $p=0.036$) (Figures 12A-12D and Figures 13A-13B). Both LV systolic performance and diastolic performance, as assessed by maximum and minimum dP/dt respectively, were greatest in the MSC_{vHO-1} group (maximum dP/dt $5717.1 \pm 935.4 \text{ mmHg/s}$, minimum dP/dt $-4893.3 \pm 1435.1 \text{ mmHg/s}$), indicating that both systolic and diastolic functions were best preserved in the MSC_{vHO-1} group after myocardial infarction (maximum dP/dt, $P=0.11$ versus MSC_{vlacZ}, $P=0.002$ versus MSCs; minimum dP/dt, $P=0.01$ versus MSC_{vlacZ}, $P=0.006$ versus MSCs). These indicators were better in the MSC_{vlacZ} (maximum dP/dt $4336.0 \pm 515.1 \text{ mmHg/s}$ $P=0.14$ versus medium group, minimum dP/dt $-3642.1 \pm 667.0 \text{ mmHg/s}$ $P=0.13$ versus medium group) and MSCs group (maximum dP/dt $3988.4 \pm 450.1 \text{ mmHg/s}$, minimum dP/dt $-3559.0 \pm 713.0 \text{ mmHg/s}$ $P=0.21$ versus medium group) than in the medium group (maximum dP/dt $3004.6 \pm 362.7 \text{ mmHg/s}$, minimum dP/dt $-2447.2 \pm 621.9 \text{ mmHg/s}$), suggesting that MSCs implantation could preserve cardiac function after myocardial infarction (Figures 15A-15D).

Example 6—Vigilant Stem Cell Transplantation in Human Subjects

Marrow isolation: Bone marrow is isolated from the iliac crest of patients with coronary artery disease after informed consent. Mononuclear cells in the bone marrow sample are isolated by Ficoll-density gradient centrifugation. The nucleated cells are centrifuged for 5 minutes at 2000 rpm ($447 \times g$), the cell pellet is resuspended, and the

cells are adjusted to a concentration of 5×10^7 nucleated cells per ml in DMEM medium supplemented with 20% FBS and 100U/ml penicillin G and 100ug/mg streptomycin). The cells are placed into a 75cm^2 corning flask and cultured in a humidified incubator at 37°C with 5% CO_2 .

5 MSC Isolation and Expansion: In the cultures, the medium is replaced every 4 days, keeping the adherent cells and discarding nonadherent cells. Each primary culture is passaged to two new flasks when the MSCs grow to approximately 70% confluence. After a series of passages, homogeneous MSCs devoid of hematopoietic cells are collected and used for cell transfection. MSCs are genetically modified with AAV-based
10 vectors, cloned, expanded, and labeled as in Example 1.

Delivery of Vigilant Cells: Vigilant cells can be delivered (*e.g.*, injected) directly into the peripheral infarct zone for myocardial regeneration, *e.g.*, when MI patients undergo surgical treatment such as coronary artery bypass or interventional treatment such as percutaneous transluminal angioplasty (PTCA). For example, coronary artery
15 bypass grafting (CABG) is performed in a coronary artery disease (CAD) patient. Once all bypass-to-coronary-artery anastomoses are completed, a vigilant cell suspension (eight injections containing autologous vigilant cells in 0.5mL, total 4×10^8 cells in 4 mL) is injected into the peri-infarct zone using a 27-gauge needle. The operation is completed as is standard in the art.

20 The present inventors have demonstrated that transplantation of vigilant HO-1 vector-engineered MSCs can reduce infarct size and improve cardiac function after left coronary artery occlusion. Moreover, it has been shown by the experiments herein that the grafting of MSCs expressing vigilant HO-1 provides advanced benefits in preserving graft MSCs and reducing infarct size. These effects were probably due to reduced
25 apoptosis in grafted MSCs and host heart. These data show that transplantation of vigilant HO-1-MSCs could be of significant value in treating acute myocardial infarction.

The acute donor cell death that occurs immediately after engraftment is thought to have a major negative impact on the ensuing graft size. It is imperative to identify the basis for the grafted cell death and to develop strategies aimed at limiting the process
30 (Reinlib, L. and Field, L. *Circulation*, 2000, 101:E182-E187). Implanted bone marrow stromal cells seem to be highly sensitive to hypoxic and inflammatory environment in ischemic myocardium. Moreover, majority of myocardial ischemia is characterized by

repeated ischemic bouts which will cause ischemic/reperfusion damage to implanted stem cells. So there is only marginal improvement in cardiac function after transplantation of MSCs into infarcted porcine heart (Shake, J.G. *et al. Ann. Thorac. Surg.*, 2002, 73:1919-1925). Therapeutic application of MSCs might ultimately require additional interventions to protect grafted MSCs with the ability of anti-ischemia in short-term and anti-ischemic/reperfusion damage in long-term. Accordingly, graft stem cells need to be engineered with beneficial genes to render grafted stem cells resistant to apoptosis, remain viable in ischemic tissue, and enhance cardiac repair after transplantation into the ischemic myocardium.

- 10 Exploiting cell growth and death regulatory factors to enhance proliferation or confer apoptosis resistance to donor cells is a potential way to improve cell transplant efficiency. Mangi *et al.* (Mangi, A.A. *et al. Nat. Med.*, 2003, 9:1195-1201) demonstrated that a direct intramuscular injection of 5×10^6 Aktengineered MSCs improved the function of infarcted rat hearts. Akt is a powerful survival signal in many systems.
- 15 However, the overall application may be affected by constitutively active Akt gene which increases the risk of tumorigenesis. With an increased understanding of the key role of heme oxygenase-1 (HO-1) in the adaptation and defense against cellular stress, HO-1 is a good candidate for providing a useful approach for cellular protection through anti-inflammation and anti-apoptosis mechanism. A system for modulating tissue physiology
- 20 has been developed which is based on an oxygen dependent degradation domain (ODD) from the hypoxia inducible factor-1-alpha (HIF1- α) which can sense ischemia and switch on cardioprotective gene. This gene system was employed to genetically manipulated graft MSCs, which improve autologous survival in local ischemic environment because cells integrated with vectors containing a gene switch/biosensor and a gene amplification
- 25 system to prevent or reverse tissue damage caused by disease. This system contains two vectors: A sensor vector contains a CMV promoter linked to a sequence encoding an oxygen-sensitive chimeric transactivator containing a GAL4 DBD, an oxygen-dependent degradation domain, and a p65 AD. An effector vector contains a cardioprotective gene-hHO-1 linked to a GAL4 UAS. The first vector expresses the chimeric transactivator
- 30 specifically in the graft MSCs in response to hypoxia, the transactivator binds to the GAL4 UAS in the effector rAAV vector. Binding of the transactivator to the UAS results in the expression of the hHO-1 gene. Accordingly, Grafted MSCs modified in this

manner might more efficiently supplement the function of weakened regenerated myocardium than un-modified cells because the vectors allow transgene expression in the regenerated tissue from vigilant cells to be regulated in response to a physiological signal, to be switched on or off, and to provide high enough levels to effect a desired result, *e.g.*,
5 prevention or reversal of cardiomyocyte damage. In this study, it is demonstrated that hypoxic treatment of engineered MSCs activates vigilant vector system expressing hHO-1 in MSCs and improves their survival in cell culture; Furthermore, it enhance their survival after engraftment to the ischemic myocardium.

In the early phase of myocardial ischemia, this strategy may salvage ischemic host
10 myocardium through HO-1 as mediated by anti-inflammatory and anti-apoptosis protection. In the late phase, regenerating muscle from survival MSCs could play an additional role in limiting infarct growth and improving infarcted heart function. Improving donor cell viability can not only limit infarct size, but to secrete proteins encoding cardioprotective activity to benefit a diseased heart (Koh, G.Y. *et al. J. Clin.*
15 *Invest.*, 1995, 95:114-121).

Graft MSCs require vasculature to provide blood supply for their survival. It has been demonstrated that implanted MSCs can differentiate into endothelial phenotype and enhance vascular density as early as day 5 after implantation in rat myocardial infarction model (Davani, S. *et al. Circulation*, 2003, 108(Suppl. 1):II253-II258). This short time
20 window enable the use of non-viral vectors. The use of Tf PEI has been shown to result in a transient and high-efficiency transfection, with little cell toxicity (Ogris, M. *et al. Gene Ther.*, 1998, 5:1425-1433; Kircheis, R. *et al. Gene Ther.*, 2001, 8:28-40). Nonviral vectors is safe and efficient to protect graft MSCs from short-term ischemic damage, however, long-term protection from ischemia/reperfusion damage may be problematic if
25 these systems do not integrate into MSCs genome. Viral vectors such as adeno-associated virus and lentivirus can afford long-lasting protective gene expression in infected MSCs. Therefore, new cardiomyocyte differentiated from engineering MSCs to express hypoxia inducible HO-1 would have a capability of self-protective in face of further repetitive ischemia.

30 In conclusion, the efficacy of the implantation of vigilant HO-1-engineering MSCs into a mouse model of myocardial infarction was demonstrated. It was shown that engrafted MSCs acquired auto-protective ability in short-term study. This pre-treatment

leads to higher survival of grafting stem cell and is associated with the improvement of heart function. So cellular cardiomyoplasty with pre-treated MSCs might be a viable way to protect graft stem cells.

These data demonstrate that vigilant HO-1 pretreatment can improve grafting
5 MSCs survival and improve contraction. Additional studies can be conducted to evaluate whether engineered MSCs-derived regenerated myocardium possess more antiischemic/reperfusion ability than host cardiomyocytes. To facilitate long-term studies, viral vectors can be used to incorporate the vigilant HO-1 gene into the chromosomes of the grafting MSCs. As more knowledge regarding the engineering of
10 MSCs for survival and differentiation *in vivo* in the long-term is gained, cellular therapy can be made more successful in treatment of ischemic heart disease.

Other Embodiments

It is to be understood that while the invention has been described in conjunction
15 with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

20 All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are
25 for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Claims

What is claimed is:

1. A genetically modified cell comprising:
 - (a) a first exogenous polynucleotide comprising a gene switch/biosensor, wherein said gene switch/biosensor encodes a physiological stimulus-sensitive chimeric transactivator and an operatively linked promoter; and
 - (b) a second exogenous polynucleotide comprising a gene amplification system, wherein said gene amplification system comprises a nucleic acid sequence encoding a therapeutic product.
2. The cell of claim 1, wherein said gene amplification system further comprises a GAL4 upstream activating sequence (UAS) linked to said nucleic acid sequence encoding said therapeutic product.
3. The cell of claim 1 or 2, wherein said chimeric transactivator is oxygen-sensitive.
4. The cell of claim 2, wherein said physiological stimulus is a signal associated with a pathological condition, and wherein said chimeric transactivator of said first exogenous polynucleotide binds to said GAL4 UAS of the second exogenous polynucleotide in response to the signal associated with the pathological condition, resulting in expression of the therapeutic nucleic acid sequence encoding the therapeutic product.
5. The cell of claim 4, wherein said signal is hypoxia associated with ischemia.
6. The cell of any of claims 1 to 3, wherein said gene switch/biosensor encodes an oxygen-sensitive chimeric transactivator and an operatively linked promoter, wherein said oxygen-sensitive chimeric transactivator comprises a GAL4 DNA-binding domain (DBD), an oxygen-dependent, and a p65 activation domain (p65AD); and wherein said nucleic acid sequence encoding a therapeutic product comprises a cardioprotective gene linked to a GAL4 upstream activating sequence (UAS).

7. The cell of claim 6, wherein said therapeutic product is selected from the group consisting of HO-1, superoxide dismutase, phospholamban (PLN) pre-pro-insulin, an anti-cell growth polypeptide, an anti-angiogenesis polypeptide, tPA, erythropoietin, a polypeptide with hypolipidemic activity, a polypeptide that acts on the cholesteryl ester transfer protein and lipase systems, a polypeptide that provides low density lipoprotein receptor replacement, a polypeptide that induces vascular protection and disobliteration of occlusions, and an interfering RNA molecule.

8. The cell of any of claims 1 to 7, wherein said cell comprises a viral vector comprising said first and second exogenous polynucleotides.

9. The cell of claim 8, wherein said viral vector is selected from the group consisting of a adeno-associated virus, an adenovirus, and a retrovirus.

10. The cell of claim 8, wherein said viral vector is adeno-associated virus.

11. The cell of any of claims 1 to 7, wherein said cell comprises a non-viral vector comprising said first and second exogenous polynucleotides.

12. The cell of any of claims 1 to 11, wherein said cell is a pluripotent or totipotent stem cell.

13. The cell of any of claims 1 to 11, wherein said cell is selected from the group consisting of a hematopoietic stem cell, a mesenchymal stem cell (MSC), a muscle derived stem cell, and a bone marrow mesenchymal progenitor cell (MPC).

14. The cell of any of claims 1 to 11, wherein said cell is selected from the group consisting of a muscle cell, a tubular cell of kidney, a type I pneumocyte of lung, a type II pneumocyte of lung, a ependymal cell, a cell from the subventricular zone of the central nervous system, a blood cell, a duct cell of the pancreas, an epidermal cell of the skin, an endothelial cell, a fat cell, an epithelial cell, a neurocell, and a Schwann cell.

15. The cell of claims 1 to 3, wherein said gene amplification system comprises nucleic acid sequences encoding multiple therapeutic products, wherein said therapeutic products are the same or different.

16. The cell of claim 1 or 2, wherein said therapeutic product is a polypeptide that is heterologous to said cell.

17. The cell of claim 1 or 2, wherein said therapeutic product is a polypeptide that is endogenous to said cell.

18. The cell of claim 1 or 2, wherein said physiological stimulus is selected from the group consisting of hypoxia, glucose, a tumor marker, and an atherosclerosis indicator of inflammation.

19. The cell of claim 1 or 2, wherein said physiological stimulus is selected from the group consisting of a cytokine, MCP-1, c-reactive protein, elevated triglyceride level, elevated oxidized LDL cholesterol level, elevated Lp(a) level, elevated homocysteine level, decreased HDL level, and decreased nitric oxide production.

20. The cell of claim 1 or 2, wherein said physiological stimulus-sensitive chimeric transactivator comprises glucose-sensitive element, and wherein said therapeutic product comprises pre-pro-insulin.

21. A method for modifying a tissue, the method comprising the step of: delivering to the tissue a genetically modified cell of any of claims 1 to 20.

22. The method of claim 21, wherein said tissue is selected from the group consisting of myocardium, mesenchymal tissue, pancreatic tissue, liver tissue, and brain tissue.

23. The method of claim 21 or 22, wherein said delivering is carried out *in vitro*.

24. The method of claim 21 or 22, wherein said delivering comprises delivering said cell to a subject *in vivo*.

25. The method of claim 24, wherein said subject is suffering from a condition selected from the group consisting of diabetes, cancer, stroke, pulmonary fibrosis, arthritis, atherosclerosis, and inflammation.

26. A genetically modified stem cell comprising:

(a) a first exogenous polynucleotide comprising:

- (1) a nucleic acid sequence encoding a GAL4 DNA-binding domain,
- (2) a nucleic acid sequence encoding an ODD domain polypeptide,
- (3) a nucleic acid sequence encoding a p65 activation domain,
- (4) two AAV ITRs, and
- (5) an operatively linked promoter; and

(b) a second exogenous polynucleotide comprising six copies of a GAL4 UAS, an E1b TATA element, a therapeutic gene, and two AAV ITRs.

27. A method for modifying a tissue, the method comprising the step of: delivering to the tissue a genetically modified stem cell of claim 26.

rAAV-GS-ODD



rAAV-Gene-hHO-1

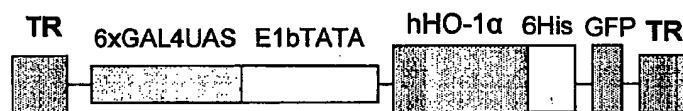


FIG. 1

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FIG. 2A

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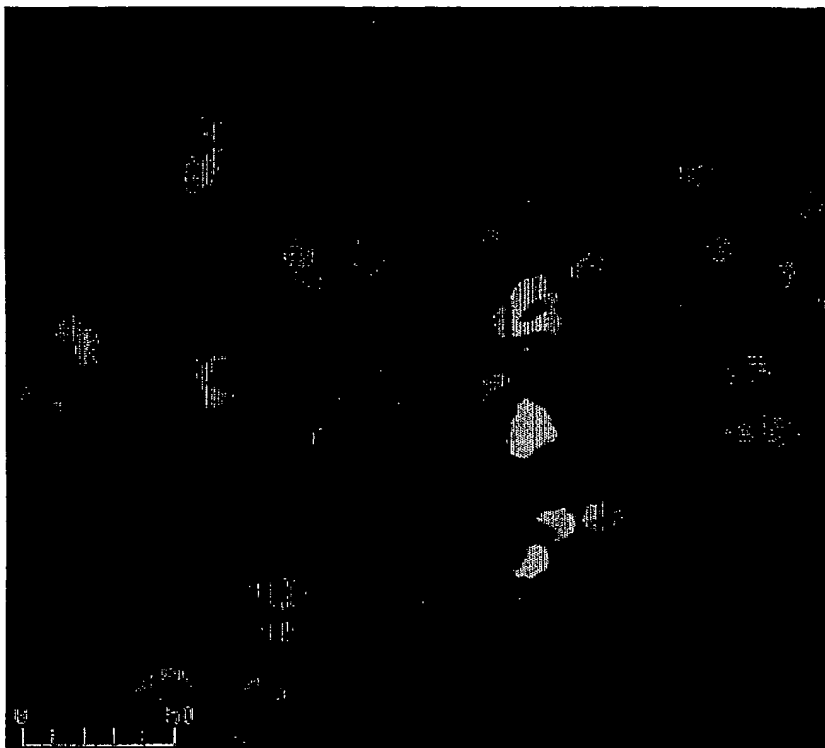


FIG. 2B

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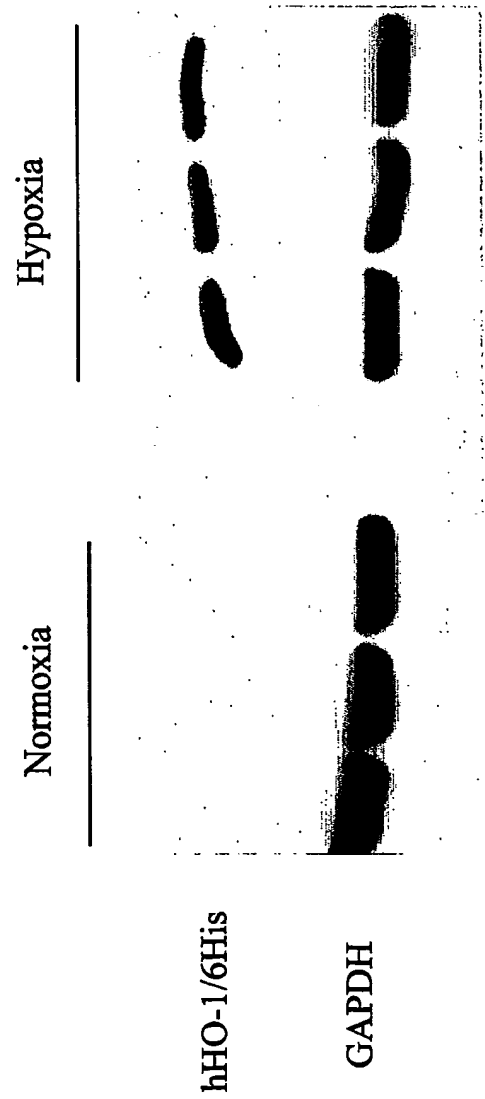


FIG. 2C

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FIG. 3A



FIG. 3B

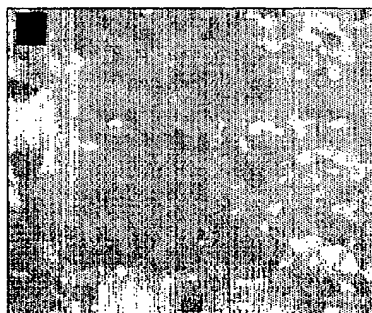


FIG. 3C

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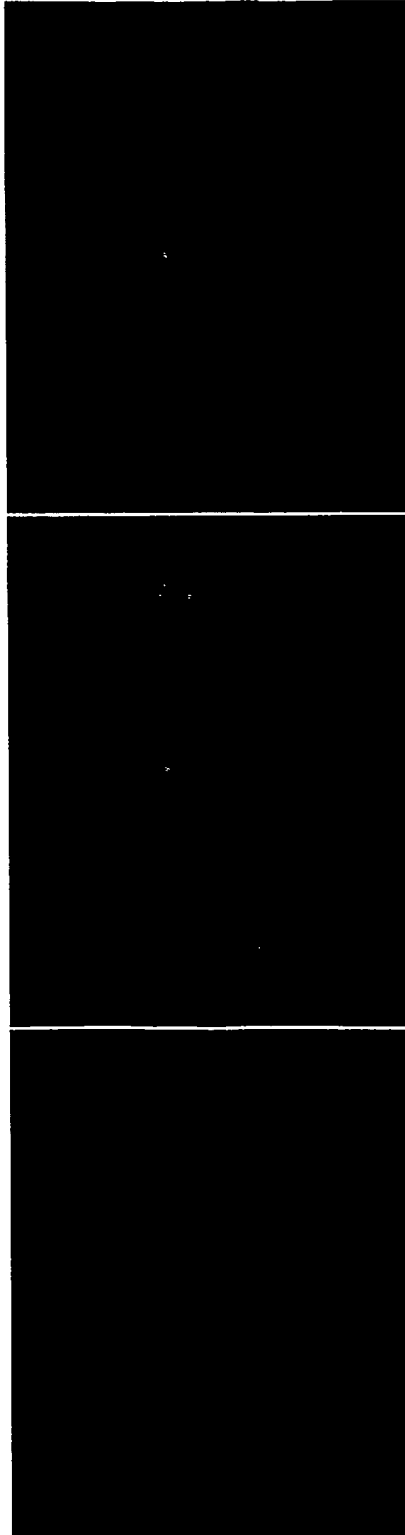


FIG. 4A

FIG. 4B

FIG. 4C

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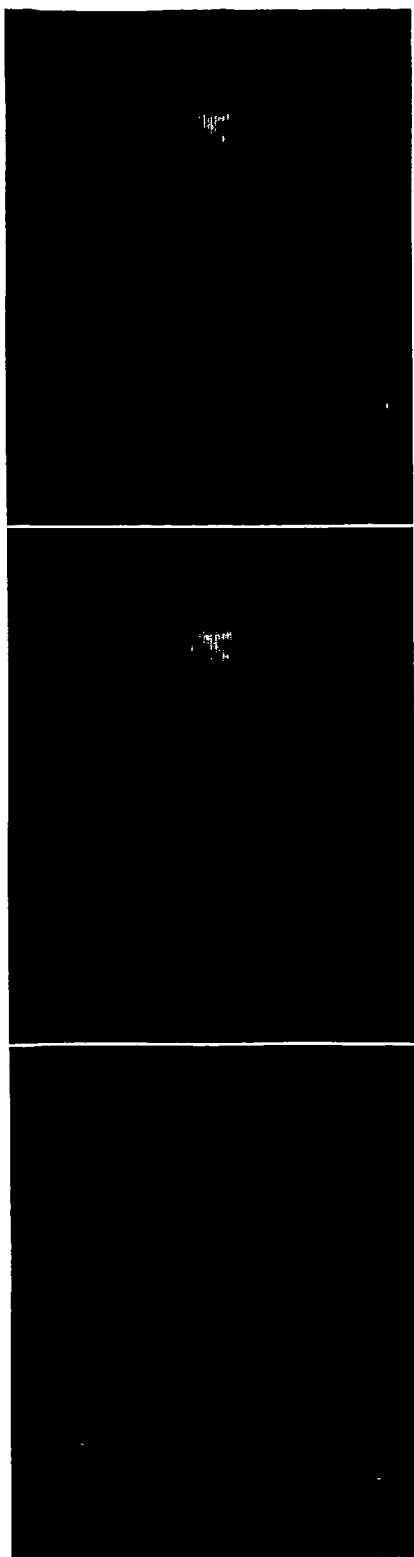


FIG. 5C

FIG. 5B

FIG. 5A

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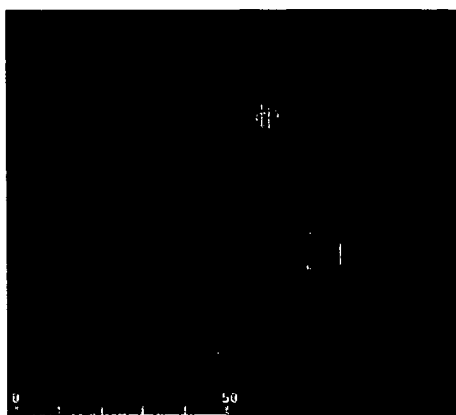


FIG. 6A

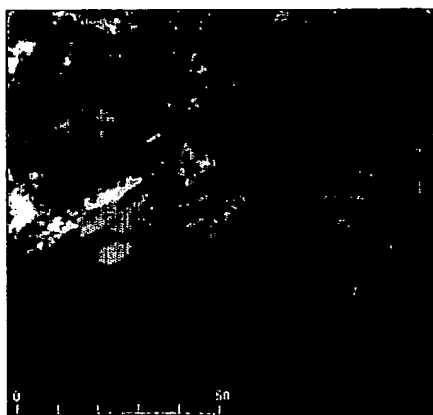
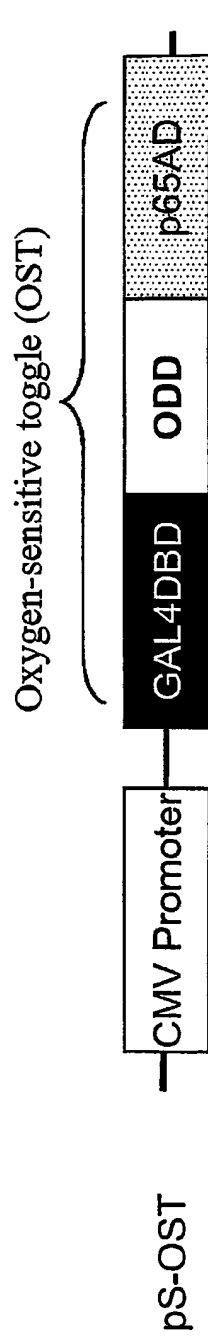


FIG. 6B

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Sensor Plasmid



Effector Plasmid

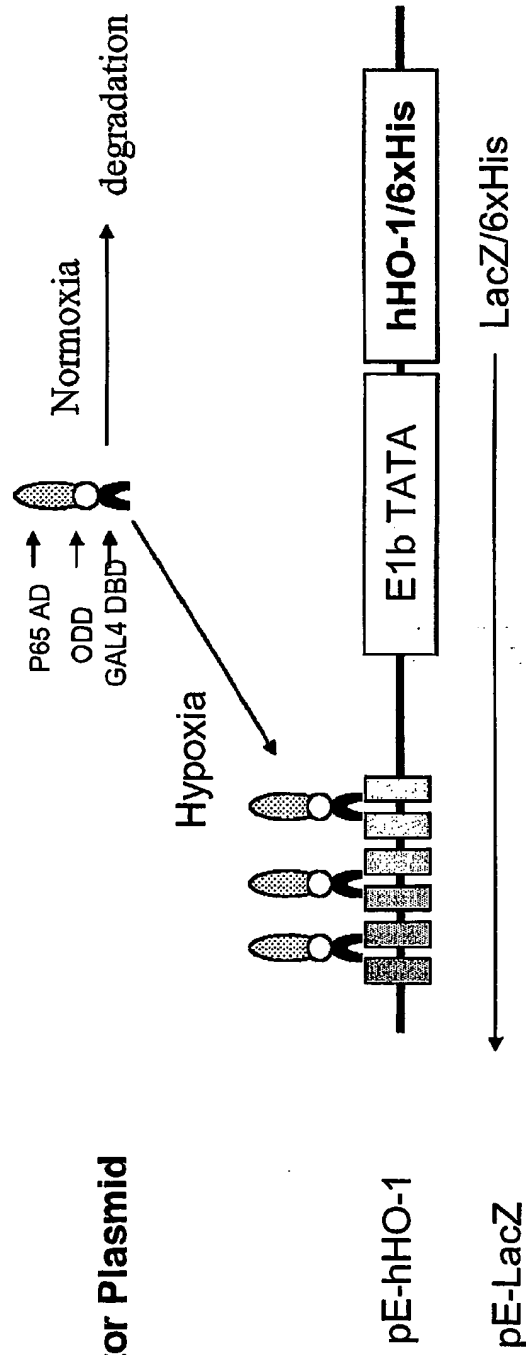


FIG. 7

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FIG. 8A

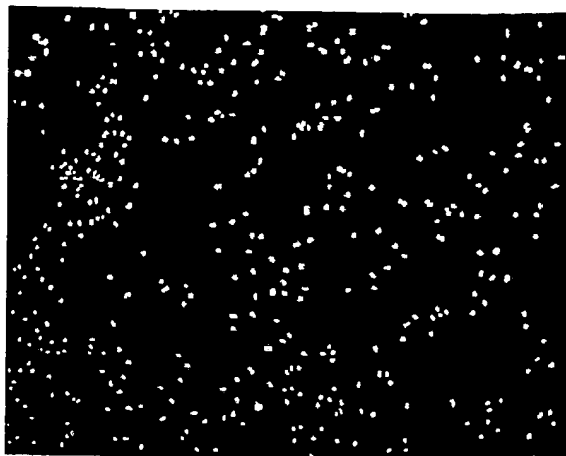


FIG. 8B



FIG. 8C



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FIG. 8D



FIG. 8E



FIG. 8F



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FIG. 8G

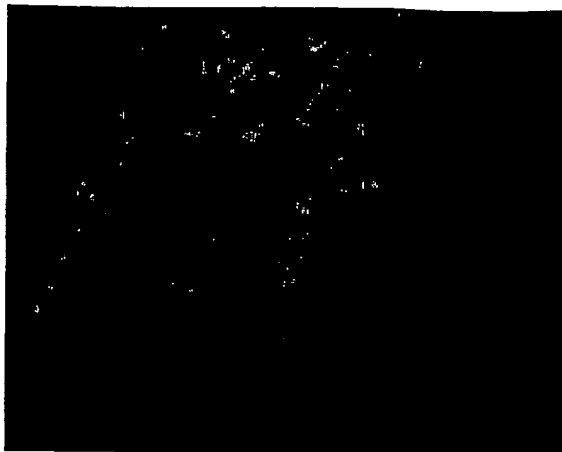


FIG. 8H



FIG. 8I



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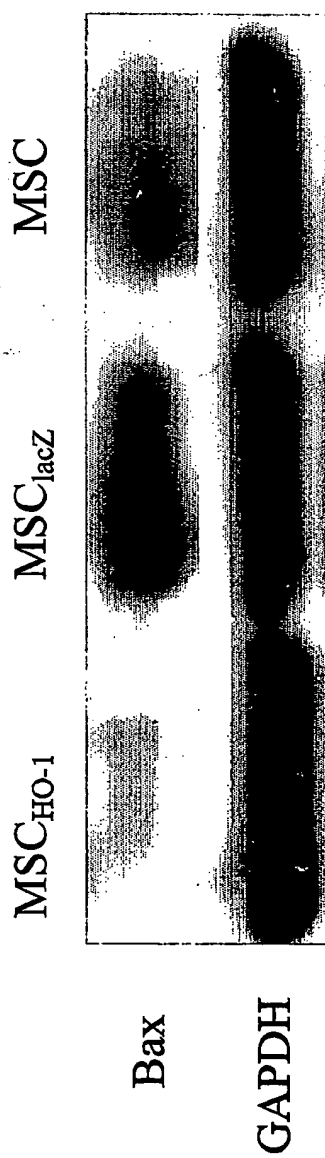


FIG. 9

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FIG. 10A

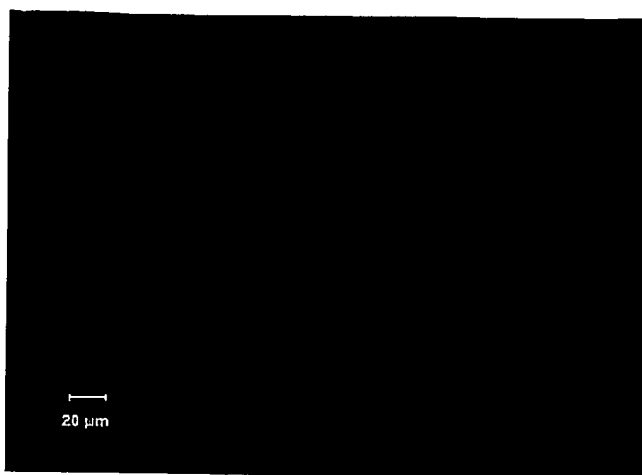


FIG. 10B

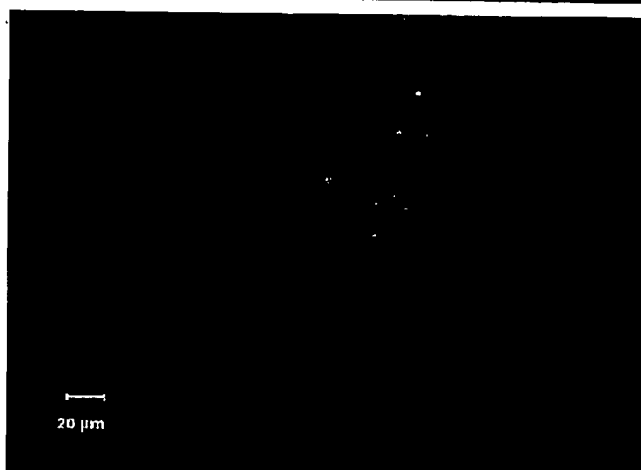


FIG. 10C



FIG. 10D

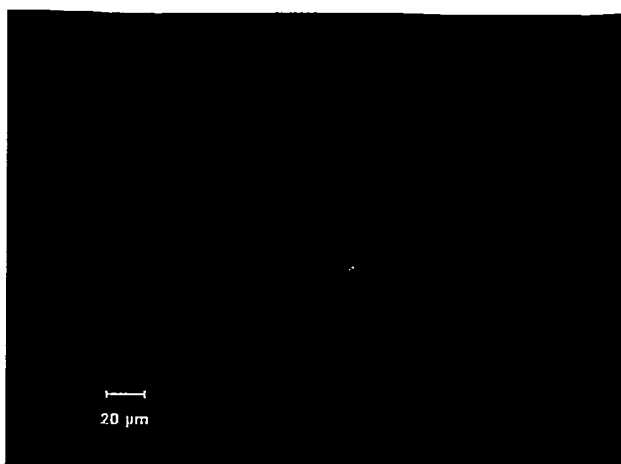


FIG. 10E

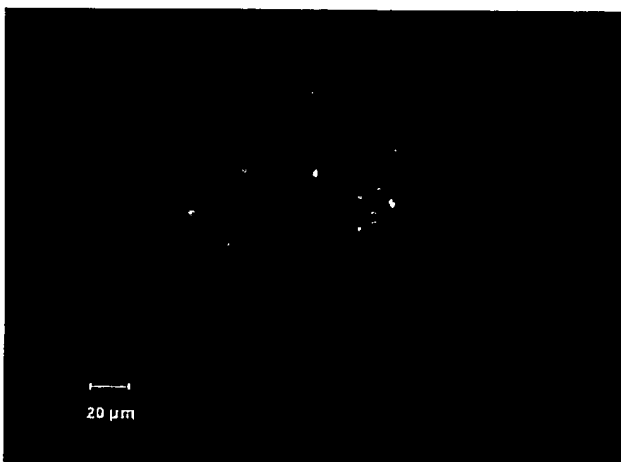


FIG. 10F

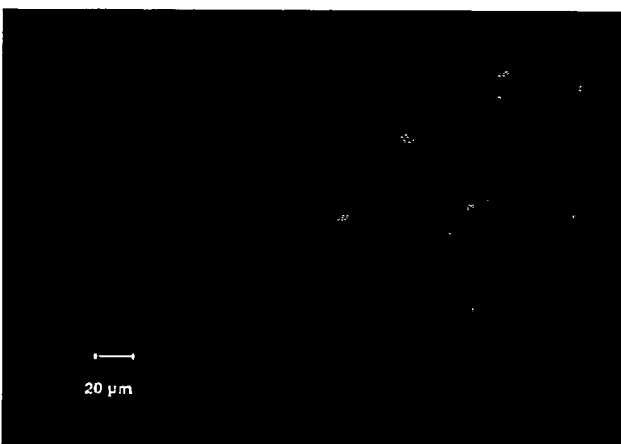


FIG. 11A

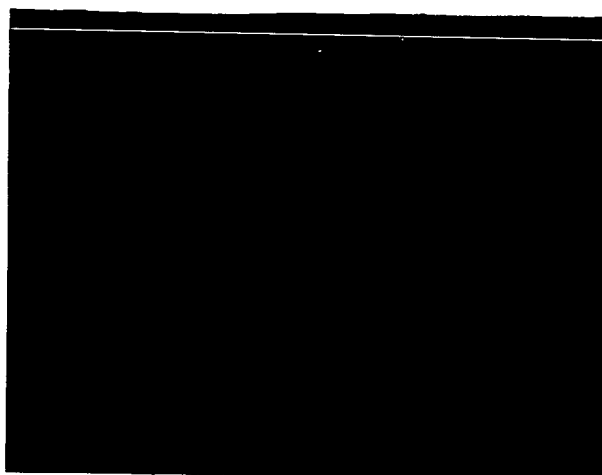


FIG. 11B

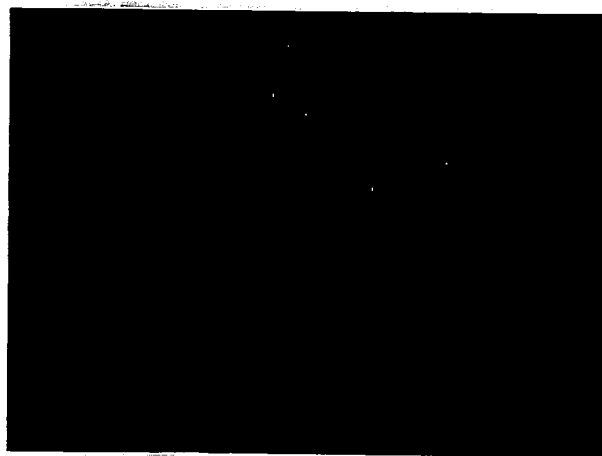


FIG. 11C

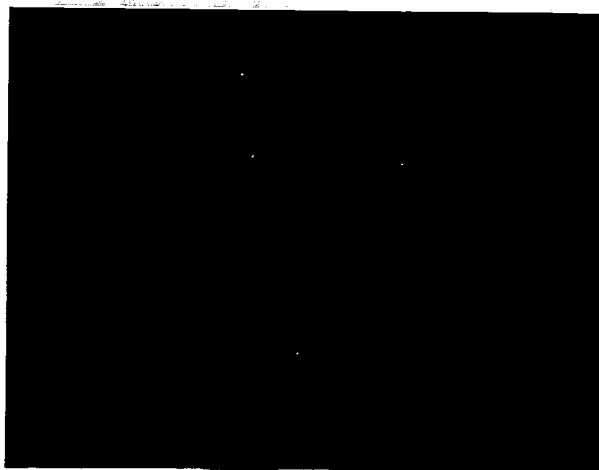


FIG. 11D

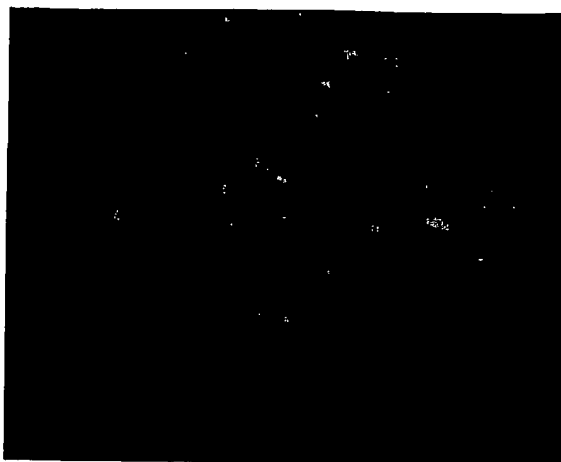


FIG. 11E

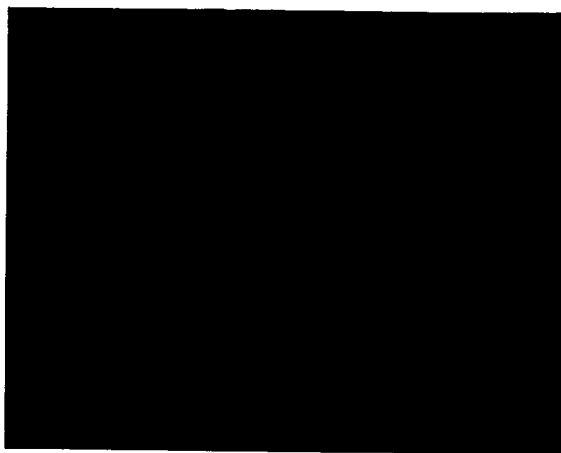


FIG. 11F



FIG. 11G

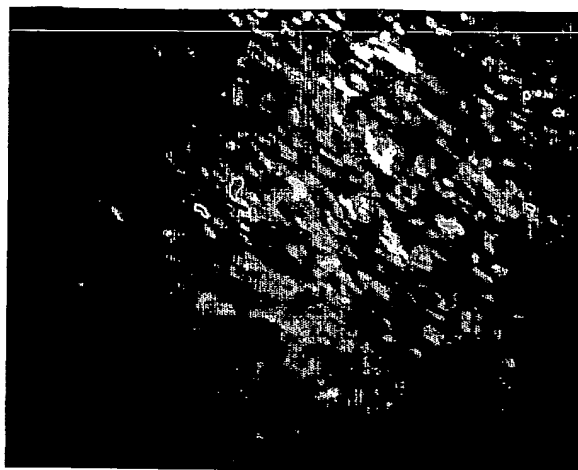


FIG. 11H

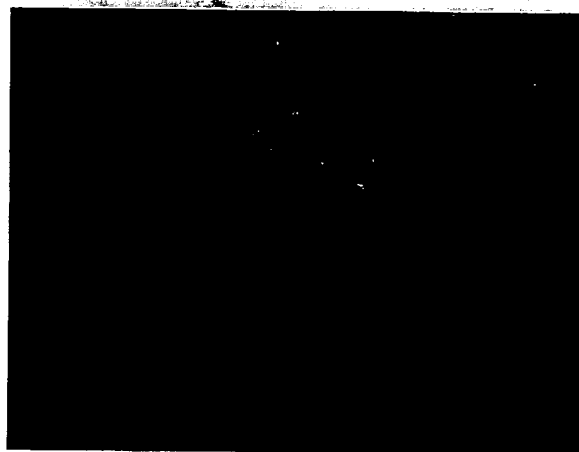
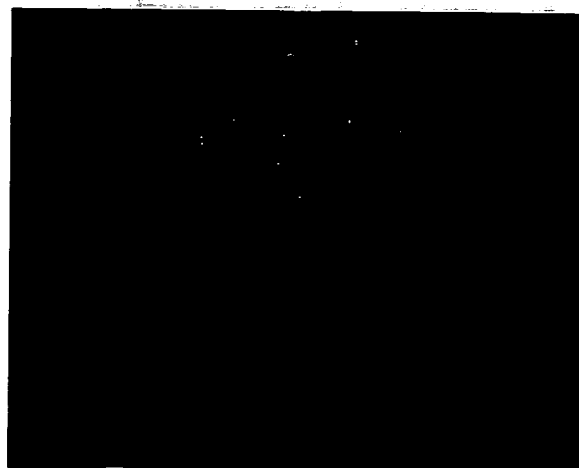


FIG. 11I



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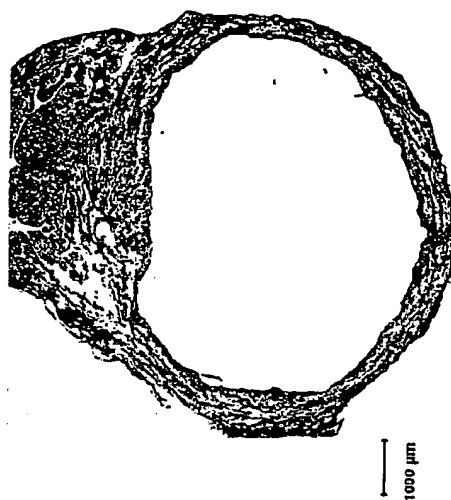


FIG. 12B



FIG. 12A

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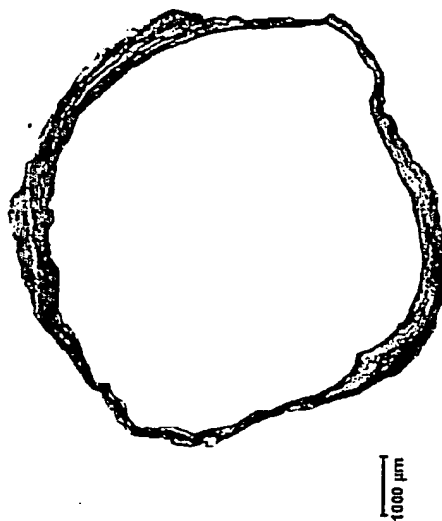


FIG. 12D

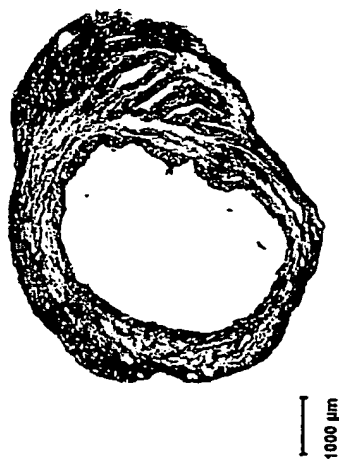


FIG. 12C

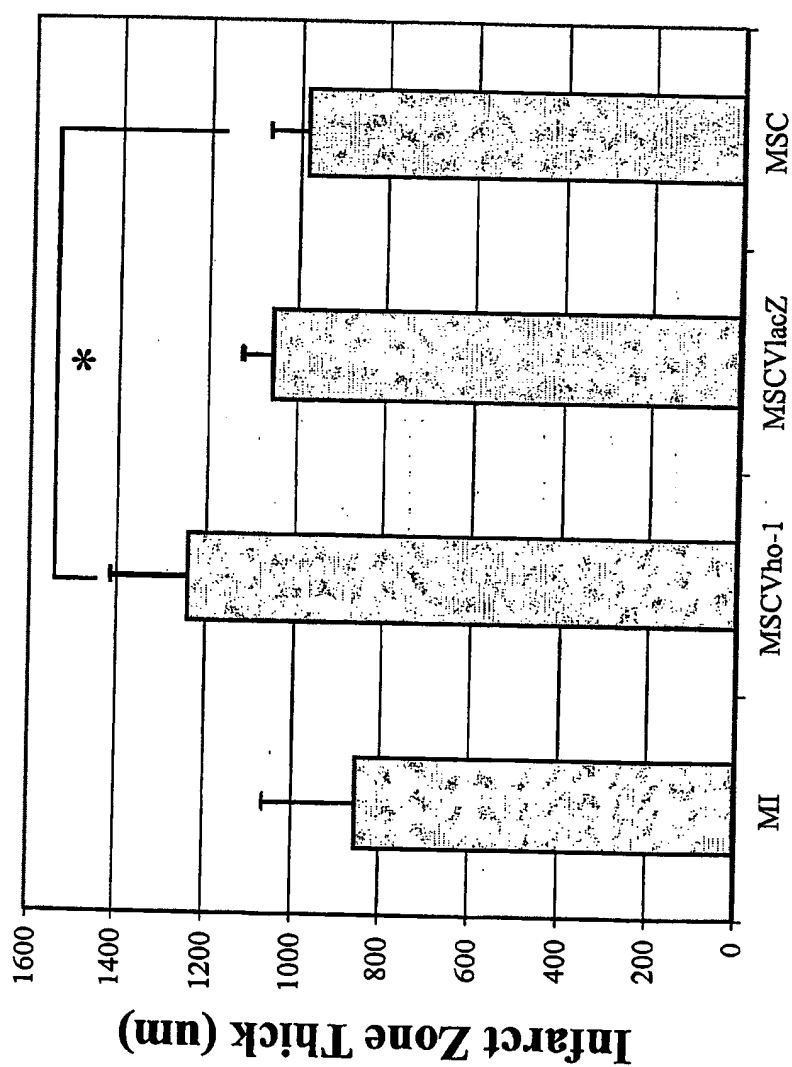


FIG. 13A

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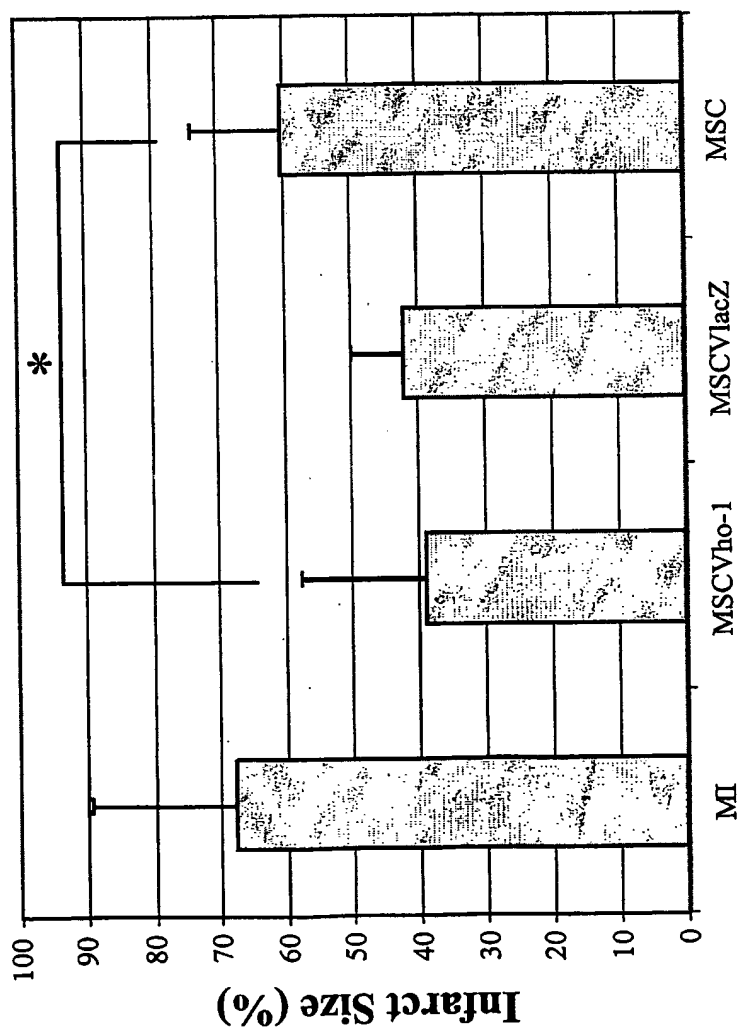


FIG. 13B

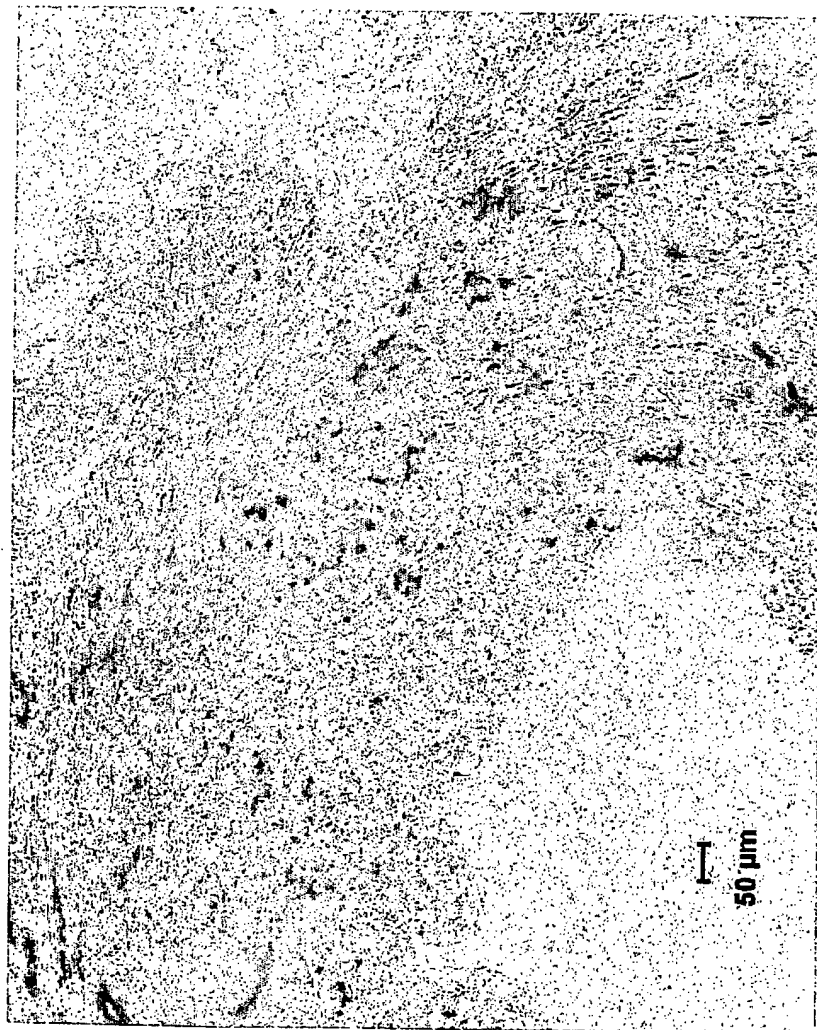


FIG. 14

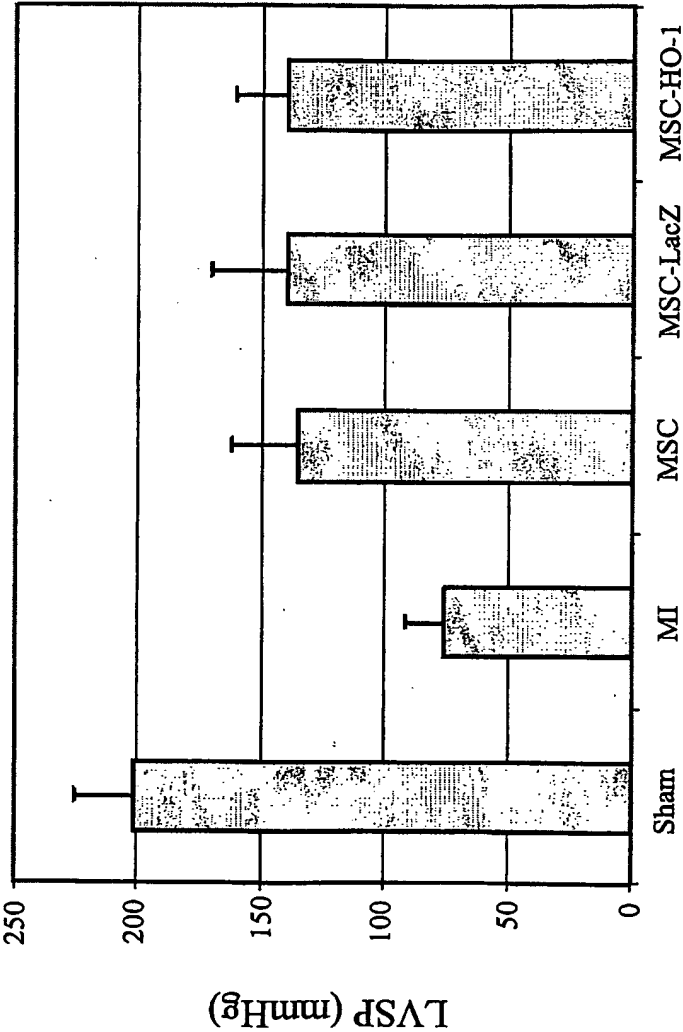


FIG. 15A

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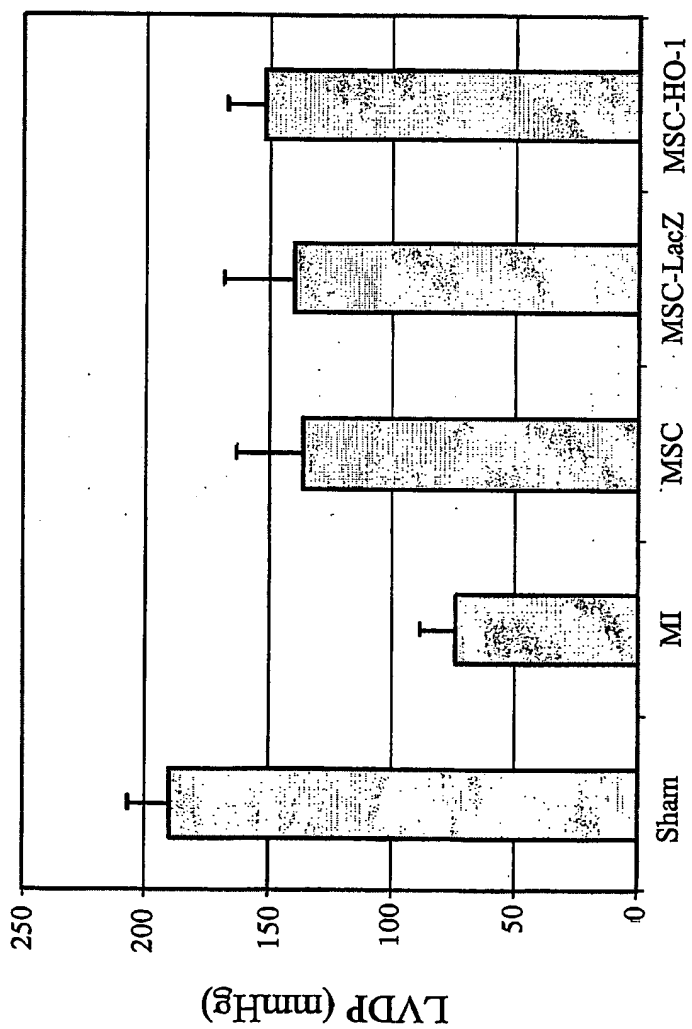


FIG. 15B

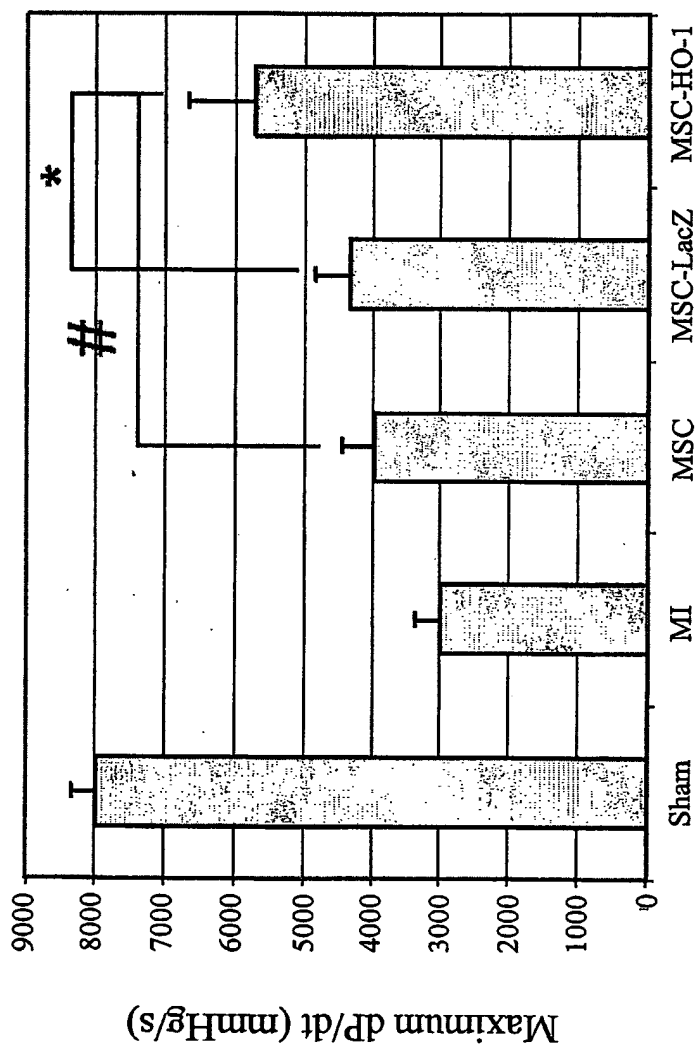


FIG. 15C

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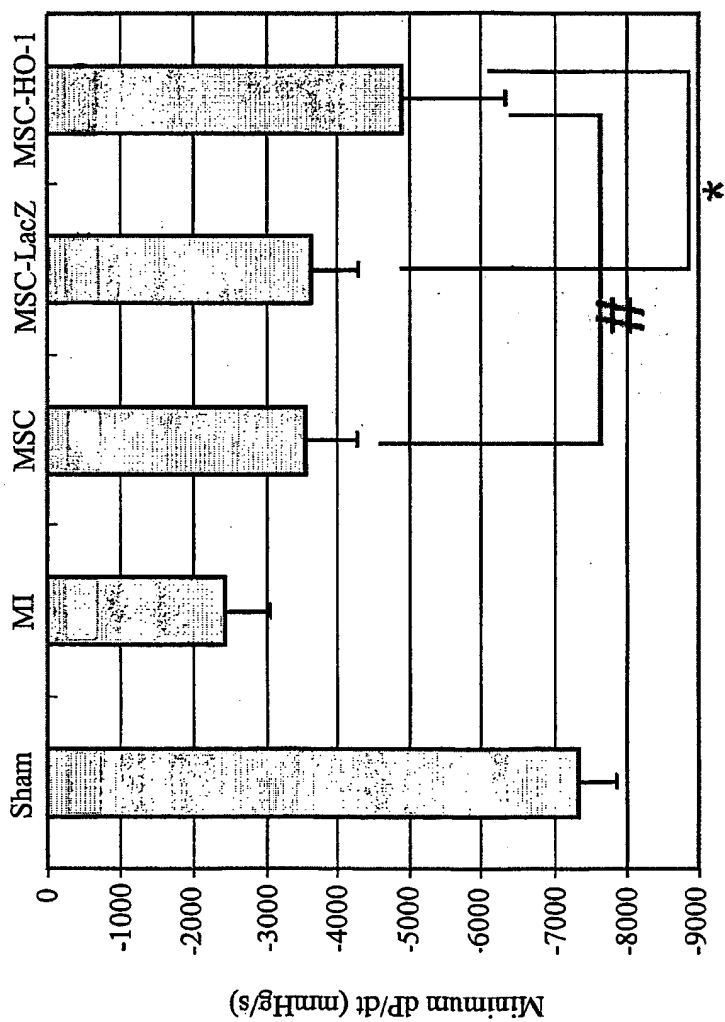


FIG. 15D

INTERNATIONAL SEARCH REPORT

International Application No

/US2004/026195

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/63 C12N15/67 C12N15/864

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANG YI ET AL: "Hypoxia inducible double plasmid system for myocardial ischemia gene therapy" HYPERTENSION (BALTIMORE), vol. 39, no. 2 Part 2, February 2002 (2002-02), pages 695-698, XP002304487 ISSN: 0194-911X the whole document ----- -/--	1-7,11, 14-18, 21-24

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

11 November 2004

Date of mailing of the international search report

26/11/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Pilat, D

INTERNATIONAL SEARCH REPORT

International Application No

/US2004/026195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PHILLIPS M IAN ET AL: "Vigilant vector: Heart-specific promoter in an adeno-associated virus vector for cardioprotection" HYPERTENSION (BALTIMORE), vol. 39, no. 2 Part 2, February 2002 (2002-02), pages 651-655, XP002304486 ISSN: 0194-911X the whole document	1-11, 14-25
X	TANG YI ET AL: "Vigilant vectors: Adeno-associated virus with a biosensor to switch on amplified therapeutic genes in specific tissues in life-threatening diseases." METHODS (ORLANDO), vol. 28, no. 2, October 2002 (2002-10), pages 259-266, XP002304488 ISSN: 1046-2023 the whole document	1-11, 14-18, 20-25
Y		12,13, 26,27
Y	PONNAZHAGAN SELVARANGAN ET AL: "Adeno-associated virus type 2-mediated transduction of murine hematopoietic cells with long-term repopulating ability and sustained expression of a human globin gene in vivo" JOURNAL OF VIROLOGY, vol. 71, no. 4, 1997, pages 3098-3104, XP002304571 ISSN: 0022-538X abstract	12,13, 26,27
A	HABERMAN R P ET AL: "INDUCIBLE LONG-TERM GENE EXPRESSION IN BRAIN WITH ADENO-ASSOCIATED VIRUS GENE TRANSISTOR" GENE THERAPY, MACMILLAN PRESS LTD., BASINGSTOKE, GB, vol. 5, no. 12, 1998, pages 1604-1611, XP000907528 ISSN: 0969-7128 abstract	
A	RUAN H ET AL: "A HYPOXIA-REGULATED ADENO-ASSOCIATED VIRUS VECTOR FOR CANCER-SPECIFIC GENE THERAPY" NEOPLASIA, DOYMA, BARCELONA,, ES, vol. 3, no. 3, 2001, pages 255-263, XP002909242 ISSN: 0212-9787 abstract	
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

/US2004/026195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH-ARICA JOSEPH R ET AL: "Switching on and off transgene expression within lactotrophic cells in the anterior pituitary gland in vivo" ENDOCRINOLOGY, vol. 142, no. 6, June 2001 (2001-06), pages 2521-2532, XP002304572 ISSN: 0013-7227 abstract -----	
A	MELO LUIS G ET AL: "Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene" CIRCULATION, vol. 105, no. 5, 5 February 2002 (2002-02-05), pages 602-607, XP002304573 ISSN: 0009-7322 abstract -----	
P,X	WO 2004/024867 A (UNIV FLORIDA) 25 March 2004 (2004-03-25) the whole document -----	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/026195

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 21-25, 27
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

/US2004/026195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004024867 A	25-03-2004	WO 2004024867 A2 US 2004161847 A1	25-03-2004 19-08-2004